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STUDIES ON TICK-BORNE ENCEPHALITIS AND OTHER ARTHROPOD-BORNE VIRUS DISEASES

Final Technical Report

By

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Abstract

Three new foci of TBE virus were located in Upper Austria.

Shrews are now essential for virus cycle in Lower Austria.

Survey with sera of game showed that TBE foci are scarcer in the West than in the East of Lower Austria. HI test was as specific but less sensitive than the NT.

Receptor-analogue substances for TBE virus are probably Ca- and Mg-salts of polyphosphoinositides.

Different strains of TBE virus induced the same level of interferon in babymouse brain. Other viruses of the TBE complex gave slightly higher interferon titers. The interferon inducing compound Poly I:C exhibited excellent protection against infection with TBE virus in mice.

Experimental studies showed that foxes, polecats and weasels can act as host of TBE virus.

The main arthropod and vertebrate hosts of Tahyna and Calovo viruses were established. Neither heterothermal nor poikilothermal vertebrates can maintain the virus cycle in winter. Overwintering of Calovo virus in Anopheles maculipennis is conceivable.

"Marburg virus" replicated in Aedes aegypti but failed to multiply in Anopheles maculipennis and in Ixodes ricinus. The virus did not induce formation of interferon in brains of baby mice and was not inhibited by Poly I:C. The agent produces CPE in ELF cells. CF test was found to be useful for diagnosis of "Marburg virus" disease.

Animals from Anatolia had antibodies against arboviruses of groups A and B.

Flow sheet, 1)

CONTENTS

TICK-BORNE ENCEPHALITIS (TBE)

Field Studies

(1) Introduction

In last year's report (Contract Number JA37-67-C-0548) investigations on the ecology of TBE virus were reported carried out in 1967 in different areas of Lower and Upper Austria, Northern Moravia and Slovakia.

Several strains of TBE virus were isolated from ticks. The role of small mammals within the cycle of TBE virus was further established by means of a serological survey.

Since Czech investigators (Kozuch,O.,Nosek,J., Lichard,M., Chmela,J. u.Ernek,E.) claim that shrews can act as a reservoir of the virus, we attempted to evaluate the significance of these animals in a focus in Lower Austria. By mark and release-trapping a home range of approximately 600 m^2 was determined and a low parasitation with ticks was found.

Finally, a survey was started with sera of game which eventually should give a complete picture of the geographic distribution of TBE virus in Austria.

In 1968, the studies were continued. Apart from the surveillance of TBE virus in Lower Austria by attempts at virus isolation from ticks collected in known foci of TBE virus, we searched for new foci in Upper Austria, where in recent years an increasing number of cases of TBE was recorded. We also pursued the studies on shrews and the survey with sera of game.

The investigations on viremia of wild-living animals after infection with TBE virus which also elucidate their role in the virus cycle in nature are reported elsewhere (see page 36).

(2) Methods

(2,1) Ticks:

Nymphs and adults of Ixodes ricinus were collected by flagdragging and transported to the laboratory. The nymphs were homogenized in pools of 10-20 individuals, the adults in pools of 5-10 individuals, respectively, suspended in a medium consisting of PBS and 10 % horse serum and inoculated intracerebrally into baby mice. The animals were observed for 14 days.

(2,2) Mark and release-trapping of shrews:

In an area of about one hectar near Hernstein small mammal traps were set up about 15 meters from each other. From March 1968 until November 1968 five excursions were done (the dates and results of excursions are listed in Table 2). The traps were baited with bread soaked in homogenized larvae of *Tenebrio molitor.* Traps were inspected two to five times during the night, captured shrews were investigated according to methods described in last year's report (Contract Number JA37-67-C-0548).

The last two excursions were done to another location about 5 km south of the above mentioned area.

(2,3) Game:

We obtained blood samples of game from different areas of Lower Austria. Sera were tested for the presence of both hemagglutination-inhibiting and neutralizing antibodies. The latter were assayed in L cells in which TBE virus was found to give a complete CPE. The cells were grown as described elsewhere in this report (see page 14). The sera were tested in a dilution 1:5 against 30-300 TCD_{50} of the virus.

(3) Results

The results of tick-collecting and of virus isolations are listed in Table 1.

(3,1) Gfieder (Lower Austria):

In 1968, the first excursion was done on May 4-5. From 659 nymphs and 50 adults no virus could be isolated. In the second excursion which was done on September 21-22, 352 nymphs and 12 adults were collected. One strain of TBE virus was isolated from a pool of 17 nymphs.

(3,2) Strelzhof (Lower Austria):

In the spring excursion in 1968 which was carried out on May 25 and 26, a total of 1083 nymphs and 213 adults were collected. No virus could be isolated. During the second excursion in autumn (September 14-15), 232 nymphs and 23 adults were collected and tested for virus: Three strains of TBE virus were isolated from pools consisting of 23 nymphs, 26 nymphs and 2 males and 20 nymphs, respectively.

(3,3) Hernstein (Lower Austria):

On June 15 and 16, 409 nymphs and 30 adults were collected. No virus could be isolated..

Five excursions for mark and release-trapping of shrews were done in this area, the results of which are shown in Table 2. In six nights (20 trap-inspections) only nine specimens of mice (Apodemus spp., Clethrionomys glareolus) and four specimens of shrews (Sorex araneus and Sorex minutus) were trapped. Also in the last two excursions done to a different area only a sparse population of shrews was observed. Because of this, shrews cannot play a major role in the virus cycle in Lower Austria and this study was discontinued.

(3.4) Upper Austria:

In recent years, we diagnosed an increasing number of cases of TBE in Upper Austria, particularly in the neighborhood of Linz. Because of this, questionnaires were sent out to patients who could frequently describe the exact location where they had picked up ticks prior to their becoming ill. On account of this information we conducted a field study from October 15-17 in three different locations (Pfennigberg/Linz, Aschach and Kronsdorf) in Upper Austria (Fig.1).

All three investigated locations were found to be foci of TBE virus. From 392 nymphs and 41 adults of Ixodes ricinus three strains of TBE virus could be isolated (Table 1).

(3.5) Game:

From 75 different locations (Fig.2) of the southwestern part of Lower Austria 236 blood samples were obtained. In Table 3 it can be seen that 22 (9%) of these specimens were positive in the HI test and 55 (23%) in the NT. It must be mentioned in connection with this discrepancy that all the sera which were positive in the HI test also gave a positive NT. Fig.3 and Table 3 show the rate of positive sera in both tests deriving from different areas. It will be noted that the percentage of positives varied from 4 to 15 percent in the HI test and from 16 to 38 percent in the NT. In Table 4 the results are listed according to the different species of game. Among roe deer and red deer approximately 8 percent positive sera were found in the HI test. This is only half of the percentage of positives found in last year's survey done with game from the southeast of Lower Austria.

(4) Discussion

It has again been shown by the results of last year's field studies that the Gfieder and Strelzhof locations harbor permanent foci of TBE virus. In coming years the surveillance of TBE in the east of Austria should, therefore, be done in these areas by attempts to isolate the virus from ticks at their peak activity in spring and in autumn.

The detection of three foci of TBE virus in Upper Austria is of particular interest. Sendung out questionnaires to patients has helped a great deal to locate these foci and this method will also be used in our future studies. Apart from Upper Austria, field studies will also be carried out in Carinthia where, because of the large wooded areas, TBE must be endemic too.

Shrews cannot play a major role in the virus cycle in Lower Austria because of their low population density registered in the Hernstein area. By contrast, the results of our experimental studies (see page 36) give conclusive evidence that some carnivora, particulary foxes, may act as reservoir of the virus.This is a new aspect on the ecology of TBE virus, Yet, the high viremia of foxes as well as high infestation with ticks combined with a home range of many square miles makes them very suited for acting as amplifying host in established foci and carrying the virus to new areas thus starting new foci. This is, perhaps, the way the virus has been or still is being introduced to the western part of Austria.

The significance of roe deer as host of TBE virus remains to be determined. As pointed out in last year's report (Contract Number DA37-67-C-0548) a final conclusion can only be drawn after the biological transmission of virus by ticks has been attempted. We are hopeful that this important study can be conducted shortly.

The results of the survey with sera of game show that foci of TBE virus are scarcer in the south west of Lower Austria as compared with the south east, the site of the previous study (Contract Number DA37-67-C-0548). It was particularly interesting to learn that the HI test is as specific as the NT but considerably less sensitive.

In surveys with animal sera the NT will pick up more positives than the HI test. This is not the case with human sera, which in our experience, give equally good results in both tests.

(5) Summary

Field studies on the ecology and geographic distribution of TBE virus were carried out in different areas.

Virus was isolated from ticks collected in the foci near Pottschach and Strelzhof (Lower Austria). Three new foci of TBE virus were found in Upper Austria (Aschach, Kronsdorf, Pfennigberg/Linz).

A low activity of shrews was observed near Hornstein. From this it appears that shrews are not an essential host of TBE virus in our foci.

Out of 236 sera of game from different locations in the southwest of Lower Austria 9.3 percent were found to possess hemagglutination-inhibiting antibodies to TBE virus, whereas 23.3 percent had neutralizing antibodies. The HI test was as specific but considerably less sensitive than the NT.

Table 1

Number of ticks (Ixodes ricinus) collected in different areas and virus strains isolated therefrom.

Excursion date	Number of nymphs collected	Number of isolated strains	Number of adults collected	Number of isolated strains
Gfieder				
May 4-5	658	-	50	-
Sept. 21-22	352	1	12	-
Strelzhof				
May 25-26	1083	-	213	-
Sept. 14-15	232	3	23	-
Hernstein				
June 15-16	409	-	30	-
Pfennigberg/Linz				
October 15 -17	35	-	10	1
Aschach				
October 15-17	190	1	19	-
Kronsdorf				
October 15-17	167	1	12	-

Table 2

Results of mark and release-trapping of shrews in Hernstein.

Excursion	Date	Number of trap-inspections per night	Number of trappings	Number of retrappings
	1968			
1	April 30 May 1	2	0	0
2	May 14-15	4	(1)*	-
3	Sept.6-7	5	(1)	-
	Sept.7-8	2	1 (1)	-
4	Oct.17-18	3	3 (2)	1
5	Nov. 9-10	4	1 (4)	-

* in parenthesis ( ) number of trappings of small mammals other than shrews.

Table 3

Serological investigations of game of 6 districts of the southwestern part of Lower Austria.

District	Number of sera tested	Number of sera pos. in the HI (%)	Number of sera pos. in the NT (%)
Lilienfeld	57	2 (3,5)	9 (15,8)
Krems (South of the Danube-River)	40	6 (12,1)	9 (27,3)
St.Pölten	33	4 (15,0)	15 (37,5)
Melk (South of the Danube-River)	42	3 (7,1)	8 (19,0)
Scheibbs	34	4 (11,8)	8 (23,5)
Amstetten	30	3 (10,0)	6 (20,0)
Total	236	22 (9,3)	55 (23,3)

Table 4

Serological investigations of different game species

Species	Number of sera tested	Number of sera pos. in the HI (%)	Number of sera pos. in the NT (%)
Capreolus capreolus	187	15 (8,0)	47 (25,1)
Cervus elaphus	33	2 (5,9)	3 (8,8)
Rupicapra rupicapra	6	3	3
Lepus europaeus	1	1	1
Vulpes vulpes	8	1	1
Meles meles	1	-	-
Total	236	22	55

Figure 1

Foci of TBE virus in Upper Austria verified through virus isolation from ticks

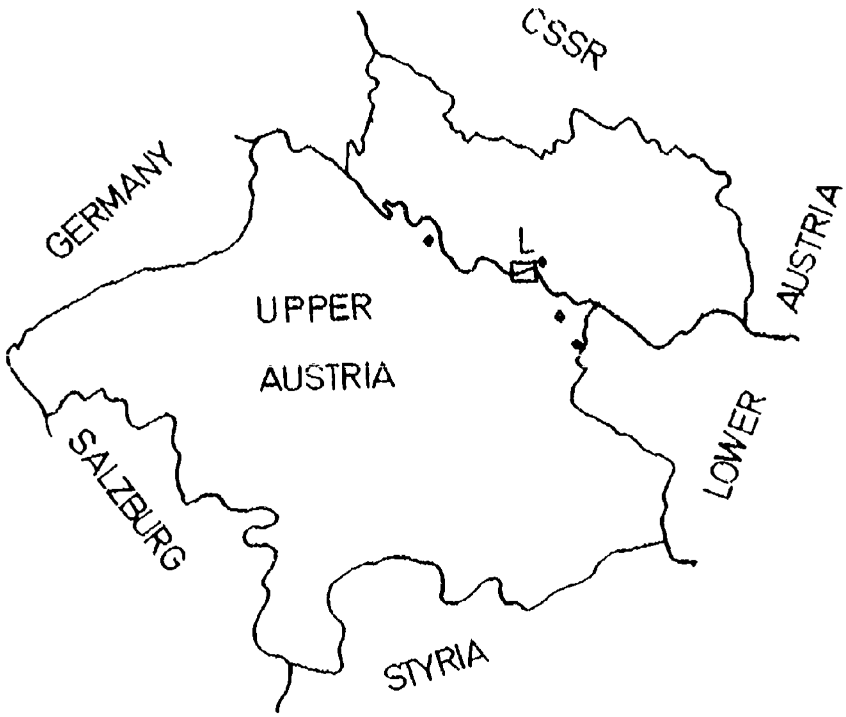
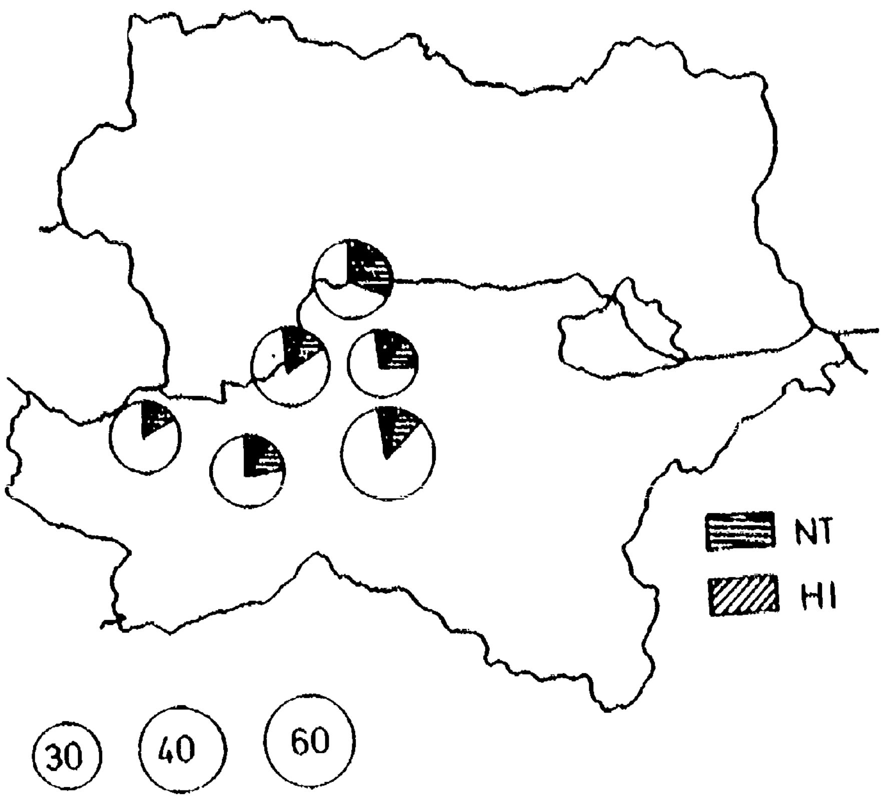


Figure 2

75 different locations in the South West of Lower Austria from which sera from game were obtained

Figure 3

Rate of sera with neutralizing and hemagglutination-inhibiting antibodies in areas under investigation



Experimental Laboratory Investigations

(1) Chemical investigations concerning receptor substances for TBE virus.

Introduction:

We found lipid substances which can competitively inhibit the hemagglutination (HA) of TBE virus and, therefore, can be regarded as receptor-analogue substances or even as the actual receptor substances. In previous experiments (1,2), we showed that small amounts of these substances could be extracted with chloroform-methanol 2:1 from brains of different animals. There, they are found in the phosphatidyl-serine fraction (3). However, the bulk of the HA-inhibiting material can best be extracted with acidified solvents. These properties, together with the observation that the activity of the substance is most strongly expressed when it is incorporated in micellar complexes with certain a trimethylammonium group-containing lipids, indicate that they might be found among the polyphosphoinositides (3).

Interpretation of earlier results:

In a previous report (2) we described a substance present in ox brain but apparently not present in mouse brain which is able to competitively inhibit the HA of TBE virus even without admixture of the mentioned basic lipids. This substance has recently been identified as a mixture of Ca- and Mg-salts of phosphatidyl-serine (4). However, in both the mentioned communications, the opinion was expressed that possibly the biological activity of this product has to be attributed not to the main substance but to an accompanying compound. In the light of the results of DAWSON (5) who could show that Ca- and in a lesser degree also Mg-salts of triphosphoinositide have a strong affinity for certain proteins and form complexes which are soluble only in solvents acidified with hydrochloric acid, our assumption that the polyphosphoinositides can act as receptor substances gains considerable weight and it is also very probable that the HA-inhibiting property of the ox brain substance is in fact due to its content of Ca- and Mg-salts of a polyphosphoinositide.

Extraction and purification of polyphosphoinositides:

Our recent work concentrated therefore on the improvement of the extraction procedures and on the identification and purification of this class of chemical compounds. By other authors (6,7,8) it has been shown that triphosphoinositide in brain is degraded to about 1/4 of its initial value whihin the first five minutes from the death of the animal, if the organ is not immediately frozen. Therefore, any brain delivered from the slaughter house did not seem to be a promising starting material. On the other hand, mouse brain, the source of active lipids in most of our earlier experiments, could not be sampled in sufficient amounts for preparing substantial quantities of polyphosphoinositides. Finally, it was possible to obtain fresh frozen brain from rhesus monkeys which were used to supply kidneys for tissue culture.

With this monkey brain as starting material, different prescriptions for the preparation of polyphosphoinositides were tried. Beginning with the rather laborious procedure of DITTMER and DAWSON (9), we obtained at first very low yields of "raw inositides". In our attempts to simplify the procedure and to increase the yield, we tried also the methods of PALMER and ROSSITER (10), DAWSON and EICHBERG (6) and SHELTAWY and DAWSON (11), all of which aim for quantitative extraction and estimation of di- and triphosphoinositide. Because the estimations of the polyphosphoinositides by all the mentioned investigators were done by determination of phosphorus and inositol, any impurities not containing these compounds did not interfere with the quantitative result. However, dark impurities which occurred in these preparations and which were degradation products of hemoglobin, occluded the hemagglutination inhibiting property of the main products and had to be eliminated. Therefore, we reverted to the original procedure of DITTMER and DASSON (9). By this method, most of the dark impurities are removed together with traces of other lipids by shaking the acidified extract with NaCl solution. After centrifuging, the dark products are left in the chloroform phase, whereas the polyphosphoinositides.. are obtained in form of an intermediate layer as their complexes with protein. These complexes are then denatured and broken with acid and the polyphosphoinositides are finally obtained as free acids of Ca-salts. By introducing some additional steps to the procedure of DITTMER and DAWSON (9), the yield could be increased, and a further addition, the extraction of an intermediate Ca-inositide-protein complex with acetone, removed the last traces of the dark impurities.

A series of small alterations of the method made it possible to adapt the volumes of solvents to the capacities of the available centrifuges. This enabled us to start with 100 g brain instead of only 70 g as before. Unfortunately, the procedure is now even more laborious and it takes about four days to prepare a sample of raw inositides from frozen brain, but now we are able to prepare in these four days 12-18 mg of a nearly colorless product instead of only 4-8 mg of a brownish substance. Our progress in the preparation of raw inositides can be seen in Table 1 and the latest procedure which incorporates also some features of the extraction procedure of WELLS and DITTMER (12) is represented in the flow sheet of Fig.1.

The material obtained with this procedure showed a high capacity for inhibiting the HA of TBE virus. It was active as low as 0.002 µg/0.4 ml when applied together with the 50 fold amount of lecithin. It contained about 10 % phosphorus which is the theoretical value for triphosphoinositide, but when tested by thin layer chromatography, it proved not to be a single substance. Therefore, further purification is necessary and the steps suggested by HERR, KFOURY and DJIBELLIAN (13) and by HENDRICKSON and BALLOU (14) will have to be considered.

<u>Chromatography of inositides:</u>

For monitoring the preparation procedure and for the estimation of the identity of intermediate and the purity of final products, a reliable method was unavailable. Our earlier attempts to develop a method of thin layer chromatography with cellulose powder did not produce very convincing results. In the meantime a TLC-method for polyphosphoinositides using silica gel H with an addition of K-oxalate was published by GONZALEZ-SASTRE and FOLCH-PI (15). However, in our hands also this method was not very satisfactory. The occurrence of a secondary front occluded the results and an identification of the spots was not unequivocal (Fig.2). A decrease of the oxalate concentration from 1 % to 0.2 % seemed to be advantageous, but neither by different activation of the plates nor by alteration of the solvent composition could this secondary front be eliminated. Also, it was not possible to let the secondary front migrate to the end of the plate by longer incubation in the tank. Finally, we had to give up this method and are now preparing formalin-treated filter paper for paper chromatography of poly-

phosphoinositides by the methods of LETTERS (16) and KAI and HAWTHORNE (17) which both treat the filter paper with formalin and acetic acid at atmospheric pressure. Their procedures are derived from the original method of HÖRHAMMER, WAGNER and RICHTER (18) who use 123° at a pressure of 18 lb/in^2. All of these methods are still used in the laboratory of DAWSON (personal communication), and we hope to be able to reproduce them and arrive at an identification of the active principle in our HA-inhibiting preparations.

Table 1: Increase of yield and quality of raw inositides from monkey brain.

Prep-No.	Starting amount	Amount of Ca-PPI-protein complex	Yield	Color	Receptor activity	Remarks	Yield/ 100 g brain
1	70 g		5.3 mg	brown	+-		7.5 mg
2	70 g	260 mg	8.8 mg	brownish	+		12.5 mg
3	100 g		4.6 mg	brownish	+		4.6 mg
4	73 g		5.7 mg	brownish	+		7.8 mg
5	100 g	380 mg	19.7 mg	dark br.	-		19.7 mg
6	102 g	307 mg	13.0 mg	yellow	+		12.8 mg
7	100 g	300 mg	8.1 mg	yellow	+	30% loss breakage	12.1 mg
8	100 g	380 mg	16.2 mg	yellow	+	20% loss spillage	20.0 mg
9	100 g	410 mg	18.5 mg	yellow	+		18.5 mg
10	100 g	440 mg	15.2 mg	yellow	+		15.2 mg
11	100 g	370 mg	12.1 mg	yellow	+		12.1 mg
12	98 g		16.2 mg	yellow	+		16.5 mg

Figure 1

Flow sheet 1)

Preparation of raw polyphosphoinositides from monkey brain.

100 g frozen brain

1) homogenize in two portions for 2 min. in waring blender with 550 ml chloroform and 400 ml methanol,
2) shake for 20 min. at room temp.,
3) centrifuge 5 min. at 2600 g.

Supernatant — Sediment

(Sediment:)
1) homogenize with 700 ml chloroform and 250 ml methanol,
2) shake 40 min. at room temp.,
3) centrifuge 10 min. at 2600 g.

Supernatant — Sediment

Combined supernatants (can be used for preparation of other phospholipids)

(Sediment:)
1) suspend in 250 ml chlorof.-methanol-1.2 N HCl 120 : 120 : 12,
2) stirr 20 min. at 39°C,
3) centrifuge 10 min. at 3500 g.

Supernatant — Sediment

(Sediment:)
1) suspend in 250 ml chloroform-methanol-0.06 N HCl 120 : 120 : 12,
2) stirr 20 min. at 39°C,
3) centrifuge 10 min. at 3500 g.

Supernatant — Sediment

(Sediment:) repeat extraction and centrifugation

Supernatant — Sediment discard

Combined acid extracts (I)
(~660 ml)

Flow sheet, contd. 2)

Combined acid extracts (I)
(~660 ml)

1) shake with 1/5 vol. 0.9 % NaCl,
2) centrifuge 10 min. at 2600 g.

Watery upper layer
discard

Lower
chloroform layer

Intermediate layer

shake again with
1/5 vol. 0.9 %
NaCl,
centrifuge

Watery upper layer
discard

Intermediate layer

Lower
chloroform layer
discard

Combined
intermediate layers (I)

1) suspend in 150 ml
chloroform-methanol 2:1,
2) shake with 30 ml 0.05 M $CaCl_2$,
3) centrifuge 10 min. at 2600 g.

Intermediate layer

Lower chlorof.
layer

Upper layer
discard

1) shake with 1/5 vol.
0.05 M $CaCl_2$,
2) centrifuge

Intermediate layer

Lower layer
discard

Upper layer
discard

Combined
intermediate layers (II)

Flow sheet, contd.3)

Combined
intermediate layers (II)

1) suspend in 50 ml acetone,
2) let stand at room temp. for 1 hr.,
3) decant.

Acetone with brown impurities
discard

Ca-Inositide-Protein Complex

1) add 50 ml acetone and reflux for 5 min.,
2) dry in vacuum at 40°C,
3) add 50 ml ethanol and reflux for 5 min.,
4) evaporate alcohol and dry in vacuum.

Denatured Ca-Inositide-Protein Complex (400 mg)

1) suspend in 75 ml chloroform-methanol 0.6 N HCl 50:25:3.75,
2) extract 15 min. at 39°C,
3) filter through glass wool.

Filtrate

Residue

repeat extraction and filtration

Filtrate

Residue
discard

Combined
acid extracts (II)
(150 ml)

1) shake with 30 ml 1 N HCl
2) centrifuge 5 min. at 3500 g.

Upper layer
discard

Intermediate layer

Lower layer

combine interm. and lower l.

Flow sheet contd. 4)

Combined intermediate
and lower layers

1) shake with 1/2 vol.
chloroform-methanol-
N HCl 3:48:47,
2) centrifuge 5 min.
at 3500 g.

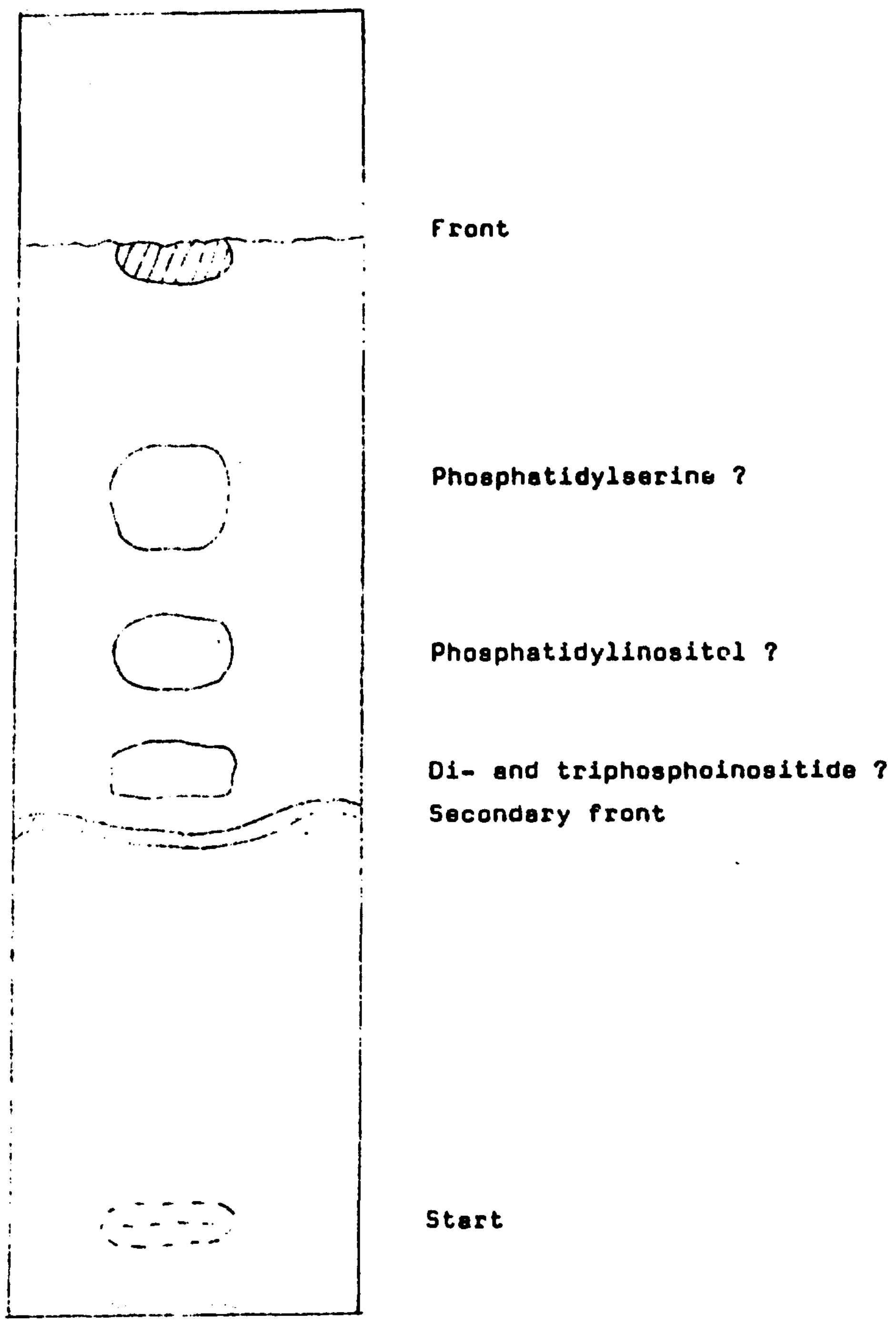
Lower layer: Raw inositides as acids in chloroform	Intermediate protein layer discard	Upper layer discard

1) wash 3 times with 0.4 vol.
0.05 M $CaCl_2$-methanol-chloroform 47:48:3
2) dry in vacuum at 40°C.

Ca-salts of
raw inositides
(12-18 mg)

Figure 2

Thin-layer chromatography of 100 µg raw inositides on silica-gel with 0.2 % K-oxalate. Solvent: chloroform-methanol-4 N NH_3 9 : 7 : 2. Time of run: 40 min.
Staining: Iodine vapour.



Summary

A class of lipid substances, extractable from fresh frozen brain, can competitively inhibit the hemagglutination by TBE virus. They can be regarded as receptor substances and are very probably the Ca- and Mg-salts of polyphosphoinositides. The procedure of the extraction of these compounds is extensively described and the increase of yield and purity by certain modifications of the process and also some experiences of thin-layer chromatography are reported.

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(2) Formation of interferon in the brain of baby mice after infection with viruses of the TBE complex.

Viruses of the TBE complex are so closely related that differentiation has been possible only with few serological methods. In particular, Central European Encephalitis (CEE) and Russian Spring Summer Encephalitis (RSSE) viruses are almost indistinguishable from each other so that they are now considered as being subtypes of the same virus, namely TBE virus.

In the present study we investigated the ability to induce interferon in baby mouse brain of the following viruses of this complex: CEE virus (strains Hypr, Jezek, Vie 415 B and Ix 22792) and one strain each of RSSE virus, Louping ill (Li) virus, Omsk hemorrhagic fever (OHF) virus, Kyasanur Forest disease (KFD) virus and Langat virus.

Baby mice were infected with 100 LD_{50} of virus. With all viruses the mice were in a moribund state five days after infection when the brains of four mice were removed and processed to extract interferon as described elsewhere in this report (see page [illegible]). However, in this experiment the interferon containing preparations were tested to prevent infection in L cells of 100-300 TCD_{50} of both EMC virus and Vesicular stomatitis virus.

As it is shown in Table 1 all viruses tested induced high levels of interferon. Suspension of brains infected with strains of CEE virus as well as with RSSE virus inhibited both challenge viruses up to a dilution of 1:320. Li, OHF, KFD and Langat virus induced four-fold more interferon. Thus RSSE and CEE viruses were not separable from each other with respect to their interferon-stimulation characteristics.

Table 1

Content of interferon in baby mouse brain

Virus	Baby mouse passage		Interferon titer against challenge virus EMC	VSV
CEE				
Hypr	9	(Vienna)	1:320	1:320
Jezek	3	(Vienna)	1:640	1:320
VIE 415 B	11		1:320	1:160
lx 22792	3		1:320	1:320
RSSE	13	(Vienna)	1:320	1:320
LI	3	(Vienna)	1:1280	1:1280
OHF	4	(Vienna)	1:1280	1:640
KFD	4	(Vienna)	1:1280	1:1280
LANGAT	3	(Vienna)	1:1280	1:640

Summary

Four strains of CEE virus as well as one strain of RSSE virus. induced the same level of interferon in baby m use brain. Fourfold higher titers of interferon were detectable in baby mouse brains after infection with one strain each of Louping ill, Omsk hemorrhagic fever, Kyasanur forest disease and Langat viruses which are also members of the TBE virus complex.

(3) Influence of the interferon-inducing compound Poly I:C on the infection with TBE virus in mice

In a series of experiments we tested the sensitivity of TBE virus to Poly I:C (Miles Chem.Corp.), a double-stranded polyribonucleotide, which is capable of inducing the formation of interferon as demonstrated by several workers.

In each experiment 80-100 mice weighing 10 g were infected subcutaneously with the Hypr strain of TBE virus. Half of the mice were given Poly I:C (dissolved in PBS at a concentration of 1 mg per ml) intraperitoneally while the other half only received PBS.

Number and time of injections with Poly I:C and amount of the drug and of virus given in each test can be seen in the Tables.

It will be seen in Table 1 that an excellent protective effect was achieved with two doses of Poly I:C (each 100 μg per mouse) provided that the treatment was started not later than three hours after infection.

For full protection of mice against encephalitis one single dose of 100 μg Poly I:C was not sufficient. This is clearly indicated by the results of two experiments shown in Table 2.

Protection of mice was achieved against low doses of virus (10 LD_{50}) only. Treated mice infected with 43 and 67 LD_{50} respectively succumbed encephalitis. However, these animals survived significantly longer than the untreated controls, thus showing that the drug still had an inhibitory effect on the infection.

From all tests mice, which had survived infection due to treatment with Poly I:C, had not acquired immunity against TBE virus and were susceptible to challenge infection.

As it was possible to inhibit TBE virus replication by application of the interferon-inducing drug Poly I:C, it can be hoped, that many other arboviruses are sensitive to this substance.

Summary

Poly I:C was capable of protecting mice against fatal tick-borne encephalitis, provided that application started befor or few hours after infection and the infactive dose was low. If therapy began later or a virus dose of approximately 50 LD_{50} was given, infected mice survived longer than untreated controls but no full protection was achieved. Mice which had survived infection due to application of Poly I:C exhibited no immunity and were susceptible to challenge infection.

Table 1

Dependance of Poly I:C influence on the beginning of treatment

Treatment Time	Treatment Dose	Number of infected mice	Number of surviving mice	Virus dose
18^h before 3^h after infection	100 µg mouse 200 µg mouse	50	47 (94%)	5 LD_{50}
0		50	10 (20%)	
18^h before 3^h after infection	100 µg mouse 100 µg mouse	50	42 (84%)	7 LD_{50}
0		50	13 (26%)	
3^h and 18^h after infection	100 µg mouse 100 µg mouse	50	34 (68%)	10 LD_{50}
0		50	2 (4%)	
24^h and 48^h after infection	100 µg mouse 100 µg mouse	50	3 (6%)	14 LD_{50}
0		50	2 (4%)	

0 = Untreated controls

Table 2

Importance of the number of injections with Poly I:C

Treatment Time	Treatment Dose	Number of infected mice	Number of surviving mice	Virus dose
3^h and 18^h after infection	100 µg mouse 100 µg mouse	50	34 (68%)	10 LD_{50}
0		50	2 (4%)	
3^h after infection	100 µg mouse	50	4 (8%)	43 LD_{50}
0		50	0	
3^h after infection	100 µg mouse	50	6 (12%)	67 LD_{50}
0		50	0	

0 = Untreated controls

Table 3

Influence of Poly I:C against different doses of virus

Virus dose	Treatment Time	Treatment Dose	Number of infected mice	Number of surviving mice	Average survival time
10 LD_{50}	3^h and 18^h after infection	100µg mouse 100µg mouse	50	34 (68%)	12.05 days
	0		50	2 (4%)	8.79 days
390 LD_{50}	3^h and 18^h after infection	100µg mouse 100µg mouse	40	3 (7,5%)	9.78 days
	0		40	0	7.88 days
3900 LD_{50}	3^h and 18^h after infection	100µg mouse 100µg mouse	40	0	8.70 days

0 = Untreated controls

(4) Viremia of white mice after infection with TBE virus

Viremia of mammals is an important factor in the ecology of arboviruses. Therefore we studied the influence of age on the development of viremia after subcutaneous infection with TBE virus.

White mice weighing 8-10 g (3 weeks old) and approximately 35 g (3 months old), respectively, were used as a model. From both groups (24 individuals each) one half was infected with low doses (approximately 100 LD_{50}) and the other half with high doses (approximately 1 000 000 LD_{50}) of TBE virus. Each day a different group of mice was bled. The blood was pooled and titrated intracerebrally in mice weighing 8-10 g.

As can be seen in Figure 1, low infective doses of virus induced two peaks of viremia in both young and old mice. However in young animals it developed faster and persisted at a higher level or a longer period than in old animals.

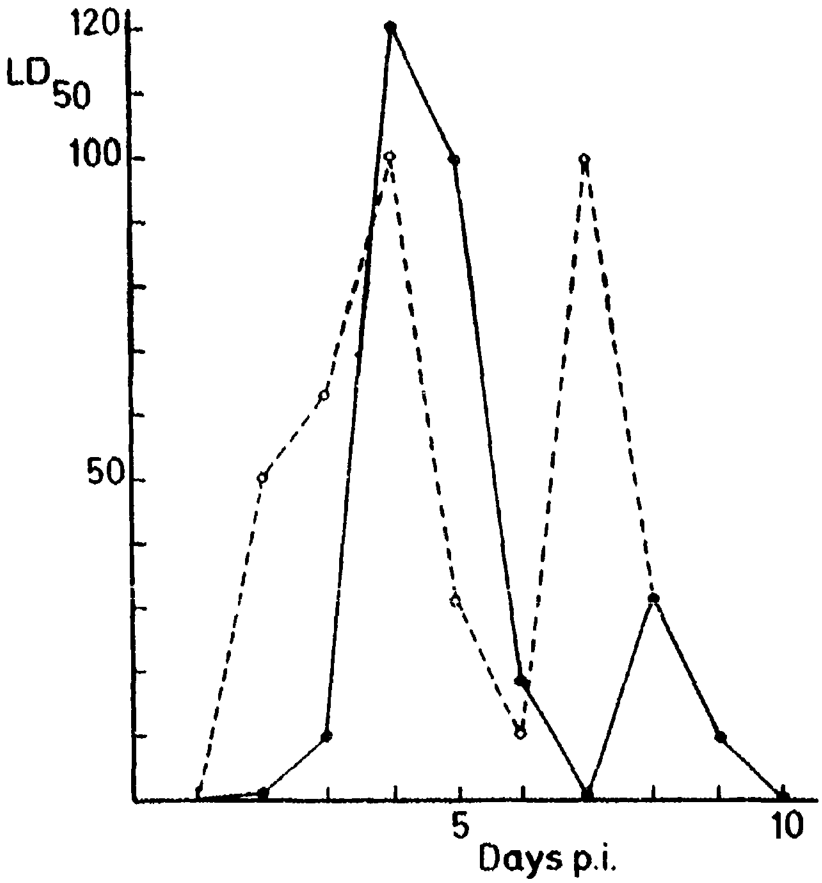
The application of a high dose of virus (Fig.2) was followed by an earlier onset of viremia as compared with those observed after a low dose. Again the 10 g mice showed higher viremia than the older mice.

From our studies it is obvious that three weeks old mice develop viremia longer and higher than adults, three months of age. Therefore it can be concluded, that young mammals are more important for the cycle of arboviruses in nature then old ones.

Summary

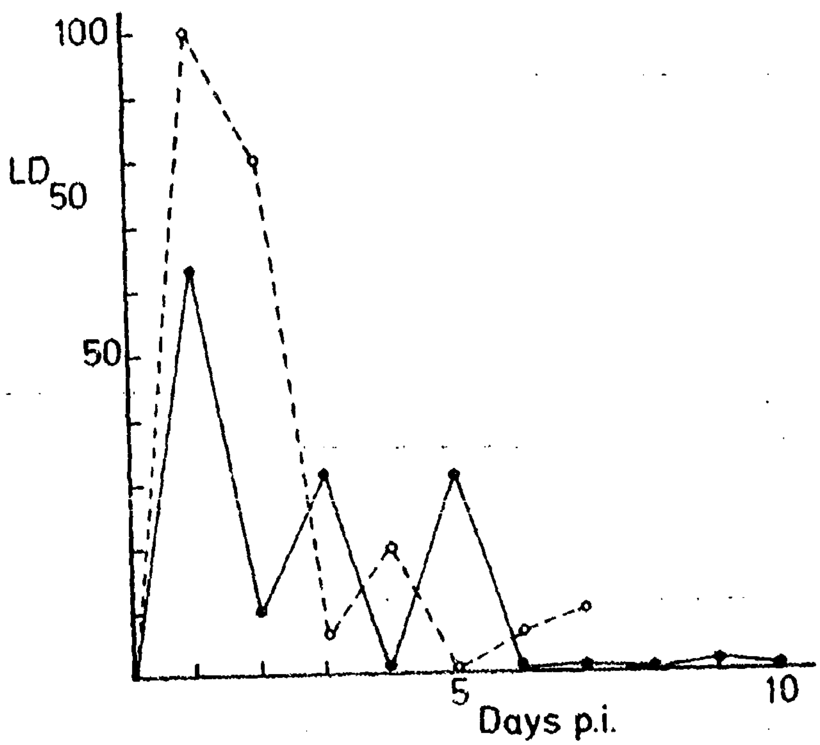
Three weeks old mice weighing 8-10 g and three months old mice weighing approximately 35 g were infected subcutaneously with 100 and 1 000 000 LD_{50} of TBE virus. Influence of age on development of viremia was studied. Low infective doses induced two peaks of viremia, both in young and adult mice, but the young ones developed viremia for a longer period. After high infective doses young animals again showed viremia longer and higher than old ones. Therefore we concluded that young mammals are more important for arbovirus cycles than old ones.

Figure 1



Viremia in young (- O - O - O -) and in adult (-● ●---●) white mice after s.c.infection with 100 LD_{50} of TBE virus

Figure 2



Viremia in young (- 0 - 0 - 0 -) and in adult (—●—●—●—) white mice after s.c.infection with 1 000 000 LD_{50} of TBE virus

(5) Viremia of some European carnivora after infection with TBE virus

In areas where TBE virus is endemic several species of carnivora are relatively abundant. In many of these animals antibodies can be found showing their susceptibility to infection. However, it is not known whether these animals reach a level of viremia which is sufficient to pass on the virus to sucking ticks and thus act as vertebrate hosts. We conducted, therefore, a number of laboratory experiments with carnivora with the hope to get some information on their potential role in the natural cycle of the virus.

Material and Methods

In the experiment, five young foxes, which were caught at an age of 5-7 weeks, three badgers and two adult weasels were employed. These animals were caught in Lower Austria. Four polecats, ten weeks old, which were obtained from a breeding station were also included in the study.

The carnivora were infected in the following manner (see also Table 1):

Three foxes (numbers 1,2 and 3), one badger (number 1), two weasels and one polecat (number 1) were infected with TBE virus by means of virophoric nymphs of *Ixodes ricinus.* These nymphs had become infected in the larval stage by gorging on viremic *Apodemus flavicollis* infected with the "Hypr" strain of TBE virus. Nymphs were used for infection about eight months after molting.

Three polecats (numbers 2,3 and 4) and two badgers numbers 2 and 3) were infected by virophoric females of *Ixodes ricinus*,which had been experimentally infected with strain "Hypr" by the insertion of a thin glass-capillary into the anal opening.

For infection, the ticks were placed inside feeding capsules which were attached with collodium to the back of the animals as described in last year's report (1).

In order to compare viremia after tha natural infection by ticks with viremia developing after the artificial infection, two foxes (numbers 4 and 5) and one badger were injected subcutaneously with high doses of virus. The strain "Jezek" used for infection of fox number 5 had originally been isolated in Czechoslovakia from a hedgehog.

The animals were bled daily for one week. The blood was tested for its content of virus in baby mice by the intracerebral route.

Sera were drawn prior to and after infection to assess the antibody response against TBE virus in the hemagglutination inhibition (HI), the tissue culture neutralization (NT) and, in some cases, in the complement fixation (CF) tests.

Results

The results are summarized in Table 1. Virus was successfully transmitted to all three foxes, four polecats and to two weasels. The three badgers showed no viremia after the setting up of ticks. The two foxes which were infected subcutaneously also developed a high viremia.

Foxes developed viremia ranging from $10^{2.5}$ LD_{50} to 10^{5} LD_{50} lasting from two to four days. It is of particular interest that the animals number 1 and 2 developed clinical symptoms of encephalitis and showed paralysis of the legs. However, the foxes did not succumb the disease and restituted completely.

The polecats showed a viremia similar to that of the foxes.

Weasel number 1 showed a short but high viremia; from the second weasel virus was isolated only from the blood sample taken on the first day p.i.

The three badgers did not develop viremia after setting up of ticks, which obviously did not suck on these mammals. We think, that the skin of badgers is too thick to be perforated by the hypostoma of ticks of the species Ixodes ricinus. After subcutaneous infection of badger number 1, virus was detectable in blood on the second day p.i. in a very low concentration only.

No antibodies were demonstrable in the serum of any animal bled on the first or second day p.i. With exception of the three badgers, all animals had acquired neutralizing antibodies after disappearance of viremia. In general, neutralizing appeared earlier than hemagglutination-inhibiting antibodies which, as it will be seen in Table 1, were not observed in all cases.

The CF test was only performed with the sera of polecats. All sera were positive 23 or 30 days, respectively, after infection.

Discussion

In a previous study, described in last year's report (1), foxes failed to develop viremia after artificial infection with 1000 LD_{50} of TBE virus. These results were somewhat puzzling because the animals also lacked a response of hemagglutination-inhibiting antibodies although this type of antibody is frequently found in sera of foxes living in foci of TBE virus. We wondered, therefore, whether the absence of viremia under laboratory conditions did not have something to do with the methods employed. In particular, we thought that if the experiments were repeated, utilyzing the technique of a biological transmission of the virus by ticks, both viremia and hemagglutination-inhibiting antibodies might be produced. This assumption was proved to be valid in the present experiments. In the light of our findings we feel that to evaluate the possible role of an animal in the cycle of an arbovirus, experiments ought to be carried out with the natural vector as done by us.

It is striking that the foxes also developed viremia after the artificial infection with 1.000,000 LD_{50} of TBE virus (as compared with 1,000 LD_{50} used in last year's experiments). This may indicate that we underestimated the amount of virus transmitted by tick bite.

According to our experiments with Apodemus flavicollis (2) on the threshold of viremia necessary for passing on the virus to sucking ticks, foxes, polecats and weasels can act as vertebrate hosts of TBE virus in nature. Carnivora are probably not necessary to maintain the virus cycle in a small focus but they may constitute amplifying hosts and, due to their large home range, carry the virus over long distances thus starting new foci.

The fact that a hemagglutination-inhibiting antibody response was not demonstrable in all animals is in good accordance with the results of the survey with sera of game. Also in this study (see page 3) more sera were positive in the NT than in the HI test.

Summary

Three young foxes (*Vulpes vulpes*), four young polecats (*Putorius putorius*), two weasels (*Mustela nivalis*) and three badgers (*Meles meles*) were infected with TBE virus by having virus-infected nymphs and females of *Ixodes ricinus* suck on them. Viremia in foxes and polecats lasted up to four days reaching maximal titers from $10.^{2.5}$ to 10^5 LD_{50}. Weasels developed viremia of a shorter duration, and no viremia was observed in badgers after the biological transmission of TBE virus.

The young foxes which were infected subcutaneously with large doses of two different strains of TBE virus ("Hypr" and "Jezek") were equally viremic as foxes infected by ticks.

Three of the infected foxes developed encephalitis within approximately two weeks p.i. but recovered. As a rule, neutralizing antibodies were found in sera after viremia had disappeared but hemagglutination-inhibiting antibodies were not constantly observed.

We concluded that carnivora can act as hosts of TBE virus in nature.

Flow sheet, 1)

Table 1

Viremia and antibody response of some European Carnivora after infection with TBE virus

Animal	Infected with	Days p.i. 1	2	3	4	5	6	7	8	9	10	11	....
F 1	10 Nymphs		x $10^{2,5}$ (-)	x $<10^{1}$	o	o (-)(..)		o			(+)(..)		
F 2	4 Nymphs	o (-)(.)	x 10^{5}	x 10^{5}	o (-)						(-)		
F 3	3 Nymphs		o (-)	x 10^{5}	x $10^{4,6}$	x 10^{4}	x 10^{2}						
F 4	"Hypr" 2,5 - 10 icLD$_{50}$ sc.	x $>10^{3}$ (-)(.)	x $10^{2,7}$	x 10^{2}	x 10^{1}								
F 5	"Jezek" 1 - 10^{5} icLD$_{50}$ sc.	x $<10^{1}$ (-)(.)	x 10^{4}	x 10^{3}	x $10^{1,9}$								
P 1	4 Nymphs	o (+)(.)	x $10^{4,5}$	x $10^{4,5}$	x 10^{5}	o +)							

Flow sheet, 1a)

Table 1

Viremia and antibody response of some European Carnivora after infection with TBE virus

		Days p.i.											
Animal	Infected with	1	2	3	4	5	6	7	8	9	10	11	....
		o	x	x	o	o							
P 2	2 Females		10^{5}	$10^{4,6}$									
		(-)(.)				(+)							
		o	x	x	o	o							
P 3	2 Females		$10^{4,6}$	$10^{4,5}$									
		(-)(.)											
		o	x	x+									
P 4	2 Females		$10^{4,7}$	$10^{4,5}$									
		(-)(.)											
			x	x	o								
W 1	4 Nymphs		$10^{4,7}$	10^{5}									
			(-)		(..)								
			x	o	o		o+						
W 2	4 Nymphs		10^{1}										
			(-)(.)		(.)								

Flow sheet, 1b) **Table 1**

Viremia and antibody response of some European Carnivora after infection with TBE virus

Animal	Infected with "Hypr"	1	2	3	4	5	6	7	8	9	10	11	....
B 1	2,5 . 10^9 icLD$_{50}$ sc.		x 10^1	o	o	o	o						
B 1	4 Nymphs	o (-)	o	o	o	o							
B 2	2 Females	o (-)	o	o	o	o							
B 3	2 Females	o (-)	o	o	o	o							

F = Fox
P = Polecat
W = Weasel
B = Badger
+ = Fatality

o = Bleeding
x = Viremia
(+)= Hemagglutination-inhibiting antibodies founc
(-)= No hemagglutination-inhibiting antibodies found
(..)= Neutralizing antibodies found
(.)= No neutralizing antibodies found

Flow sheet, contd.2) <u>Table 1</u>

Viremia and antibody response of some European Carnivora after infection with TBE virus

Animal	Infected with	Days p.i. 15	21	22	23	30
F 1	10 Nymphs					
F 2	4 Nymphs	(-)(..)				
F 3	3 Nymphs					
F 4	"Hypr" 2,5 . 10 icLD$_{50}$ sc.	(-)(..)			(-)(..)	
F 5	"Jezek" 1 . 10^5 icLD$_{50}$ sc.	(-)(..)			(+)(..)	
P 1	4 Nymphs					(+) (*)
P 2	2 Females				(+) (*)	

Flow sheet, contc.2a)

Table 1

Viremia and antibody response of some European Carnivora after infection with TBE virus

Animal	Infected with	Days p.i. 15	....	21	22	23	....	30
P 3	2 Females					(+) (*)		
P 4	2 Females					(+) (*)		
W 1	4 Nymphs	(+)						
W 2	4 Nymphs							
B 1	4 Nymphs	(-)(..)						
B 1	"Hypr" 2,5 . 10^9 icLD$_{50}$ sc.							
B 2	2 Females							

Flow sheet, contd.2b) Table 1

Viremia and antibody response of some European Carnivora after infection with TBE virus

		Days p.i.						
Animal	Infected with	15	....	21	22	23	....	30
B 3	2 Females							

F = Fox
P = Polecat
W = Weasel
B = Badger
\+ = Fatality

o = Bleeding
x = Viremia
(+)= Hemagglutination-inhibiting antibodies found
(-)= No hemagglutination-inhibiting antibodies found
(..)= Neutralizing antibodies found
(.)= No neutralizing antibodies found
(*)= Complement-fixing antibodies found

L i t e r a t u r e   c i t e d

(1) Kunz, Ch.: Follow-up Studies of Tick-Borne Encephalitis.
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(2) Radda, A., H. Hofmann, G. Pretzmann: Threshold of viremia in Apodemus flavicollis for infection of Ixodes ricinus with Tick-borne encephalitis.
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Diagnostic Studies on Patients

Diagnostic studies were done as repeatedly described in previous reports.

From January through December 1968, a total of 78 cases of TBE and 21 cases of possible TBE were diagnosed in our laboratory. The patients were hospitalized in the following Austrian provinces: Vienna 22 (7)*, Burgenland 3 (1), Lower Austria 30 (11) and Upper Austria 23 (2). The high incidence of TBE in Upper Austria is striking and could indicate the advance of the virus to the West of Europe (see also page 3).

* ( ) = probably TBE

STUDIES ON MOSQUITO-BORNE VIRUSES IN AUSTRIA

(1) Introduction

Since 1963, extensive field studies on mosquitoes and their role as vectors of arboviruses were carried out in several parts of Eastern Austria. These investigations led to isolations of Tahyna virus in the Danube valley (1,2) and in the steppe biotops east to the Neusiedlersee (2,3) and of Calovo virus (3,4) in the Neusiedlersee-area.

Due to the fact that in the Eastern part of the Neusiedlersee-area - the so-called Seewinkel - at the Hungarian border both viruses were detected in extremely high infection rates, our studies were concentrated to this area with the aim to establish the cycle of these viruses throughout the year. For this purpose, the following investigations were carried out:

1. Mass collections of mosquitoes and virus isolation experiments therefrom, in order to find the species able to transmit the viruses and to determine the months of virus activity.

2. Serological investigations on wild-living and domestic vertebrates in order to get some information on the spectrum of vertebrate hosts, particularly also on those which might act as reservoir of the viruses during winter. These studies were supplemented by studies on the course of experimental infection of several vertebrate species.

3. Exposure of sentinel rabbits in order to get further information on the seasonal occurrence of the viruses.

4. Experimental studies on the hibernation of Tahyna and Calovo viruses.

(2) Mass collections of mosquitoes and virus isolation experiments therefrom

In 1967 from April 13 to November 13, 17 excursions were carried out to the steppe biotops in the Seewinkel near the Hungarian border. Mosquitoes were regularly collected by two methods. On one hand, mosquitoes were caught in a cow barn, two rabbit cages and a pig barn during day. On the other hand, entomological nets were swept through the air from evening twilight until one hour after sunset. The mosquitoes were sucked into aspirators, immediately frozen in dry ice and then kept at -80°C until processing for virus isolation experiments. For this purpose, mosquitoes were identified under the stereomicroscope. Then they were ground in mortars and suspended in 2.5 ml of TCM 199 containing 0.75% bovine albumine and and antibiotics buffered to 7.2 pH. Pool size varied from 1 to 50 individuals. The suspensions were centrifuged at 4000 rpm for 30 minutes, and the supernatants were inoculated intracerebrally into baby mice, which mice were observed for fourteen days for signs of illness. Identification of virus strains was done by means of the immunofluorescent method (5).

The results of collections of mosquitoes and of virus isolation experiments are shown in Tables 1 and 2. It will be seen that altogether 108,207 mosquitoes comprising 19 species were caught in 1967 from which 107,832 individuals were tested for virus in 2438 pools.

A total of 25 strains of -hyna virus was isolated, namely 15 from mixed pools of _ ~~es caspius~~ and Aedes dorsalis, one from Mansonia richiardii, three from Anopheles maculipennis and six from unidentified mosquitoes. Mosquitoes infected with Tahyna virus were found during the period from June 17 to July 26.

In addition, 53 strains of Calovo virus were isolated, all deriving from Anopheles maculipennis. This virus was found during the period from August 4 to September 29.

Moreover, four agents, presumably viruses, were isolated from Aedes vexans (June 17), Anopheles maculipennis (July 14), Mansonia richiardii (July 14 and July 24), which could not be identified as Tahyna virus nor Calovo virus by means of the immunofluorescent method. Studies on these agents are in progress.

In 1968, 11 excursions were carried out in the same localities as in 1967 during the period from April 18 to October 29. A total of about 50,000 mosquitoes was collected. So far, only a small part has been tested for virus. We succeeded in isolating four strains of Tahyna virus from mixed pools of *Aedes caspius* and *Aedes dorsalis* collected on June 25, and one strain of Tahyna virus from *Anopheles maculipennis* collected on July 10.

Flow sheet, 1)

Table 1

Collections of mosquitoes in berns in the Seewinkel in 1967 and virus isolation experiments

	Date of Collection					
Species	April 13	April 21-23	May 5-7	May 19-21	June 2-4	June 17-18
An.maculipennis	25/25/1/- *)	250/250/5/-	185/185/5/-	807/807/19/-	975/975/21/-	652/652/14/-
A.claviger			3/ 3/1/-	10/ 4/ 2/-	4/ 2/ 1/-	71/ 70/ 2/-
Ae.flavescens				12/ 12/ 1/-		8/ 8/ 2/-
Ae.caspius dorsalis				12/ 12/ 1/-		203/203/ 5/-
Ae.cantens				1/ 1/ 1/-		
Ae.vexans						3/ 3/ 2/-
M.richiardii						9/ 9/ 2/-
Th.annulata	4/ 4/1/-	1/ 1/1/-	3/ 3/2/-	4/ 3/ 2/-	11/ 11/ 2/-	14/ 14/ 2/-
U.unquiculata						
C.pipiens	19/19/1/-	1/ -/ -/-		6/ 6/ 1/-		1/ 1/ 1/-
C.modestus						
C.territans	3/ 3/1/-					
Unidentified						
	51/51/4/-	252/251/6/-	191/191/8/-	852/845/27/-	990/988/24/-	961/960/30/-

Flow sheet, contd.2)

Table 1

Collections of mosquitoes in barns in the Seewinkel in 1967 and virus isolation experiments

	Date of Collection			
Species	June 30 - July 2	July 14 - 17	July 24 - 26	August 4 - 6
An.maculipennis	2868/2868/58/2 T*)	5219/5218/105/-	10144/10144/204/-	2352/2352/48/1 C
A.claviger	7/ 7/ 1/-			
Ae.flavescens				
Ae.caspius dorsalis	123/ 123/ 6/-	41/ 41/ 5/1 T	188/ 188/ 7/-	
Ae.cantans				
Ae.vexans	15/ 15/ 2/-	1/ 1/ 1/-	1/ 1/ 1/-	
M.richiardii	255/ 255/ 9/-	183/ 183/ 7/-	281/ 281/ 10/-	10/ 10/ 2/-
Th.annulata	16/ 16/ 2/-	41/ 41/ 4/-	22/ 22/ 6/-	
U.unguiculata				
C.pipiens		1/ 1/ 1/-	22/ 21/ 2/-	32/ 32/ 1/-
C.modestus	1/ 1/ 1/-	1/ 1/	1/ 1/ 1/-	8/ 8/ 2/-
C.torritans				
Unidentified	850/ 850/17/1 T	11650/11650/233/3 T		
	4135/4135/96/3 T	17137/17136/356/4 T	10659/10658/231/-	2402/2402/53/1 C

Flow sheet, contd.3)

Table 1

Collections of mosquitoes in barns in the Seewinkel in 1967 and virus isolation experiments

	D a t e o f C o l l e c t i o n			
Species	August 18 - 20	September 1 - 3	September 8 - 10	September 28 - 29
An.maculi-pennis	1039/1039/22/2 C *)	2000/2000/40/8 C	3129/3129/63/34/ C	808/808/17/7 C
A.claviger			1/ 1/ 1/-	22/ 22/ 3/-
Ae.flavescens				
Ae.caspius dorsalis	3/ 3/ 1/-	11/ 11/ 1/-	1/ 1/ 1/-	130/130/ 3/-
Ae.cantans				
Ae.vexans	1/ 1/ 1/-			2/ 2/ 1/-
M.richiardii	14/ 14/ 2/-	121/ 121/ 4/-	154/ 154/ 7/-	4/ 4/ 2/-
Th.annulata	10/ 10/ 2/-	28/ 28/ 2/-	184/ 184/ 7/-	82/ 79/ 2/-
U.unquiculata		1/ 1/ 1/-	5/ 5/ 1/-	3/ -/ -/-
C.pipiens	17/ 14/ 3/-	24/ 22/ 2/-	42/ 31/ 2/-	112/100/ 2/-
C.modestus	14/ 12/ 1/0	8/ 8/ 2/-	8/ 8/ 1/-	1/ -/ -/-
C.territans		1/ -/ -/-		
Unidentified				
	1098/1093/32/2 C	2194/2191/52/8 C	3524/3513/83/34 C	1164/1145/30/7 C

Flow sheet, contd.4)

Table 1

Collections of mosquitoes in barns in the Seewinkel in 1967 and virus isolation experiments

Species	October 16 - 17	October 29 - 30	November 12 - 13	Total
An.maculipennis	82/ 82/ 2/-*)	11/ 11/1/-	6/ 6/1/-	30552/ 30551/ 662/2 T, 52 C
A.claviger	15/ 15/ 1/-			133/ 124/ 12/-
Ae.flavescens				20/ 20/ 3/-
Ae.caspius dorsalis	92/ 92/ 2/-	4/ 4/1/-		808/ 808/ 33/1 T,
Ae.cantans				1/ 1/ 1/-
Ae.vexans	2/ 2/ 1/-			25/ 25/ 9/-
M.richiardii				1031/ 1031/ 45/-
Th.annulata	59/ 59/ 2/-	14/ 14/1/-	18/18/1/-	511/ 507/ 39/-
U.unquiculata		3/ 3/1/-		12/ 9/ 3/-
C.p piens	92/ 92/ 2/-	100/100/2/-	50/50/1/-	519/ 489/ 21/-
C.modestus	2/ 2/ 1/-	2/ 2/1/-		46/ 43/ 10/-
C.territans				4/ 3/ 1/-
Unidentified				12500/ 12500/ 250/4 T,
	344/344/11/-	134/134/7/-	74/74/3/-	46162/ 46111/1089/7 T, 52 C

*) Number of mosquitoes collected/Number of mosquitoes tested for virus/Number of pools/Number of virus Strains isolated (T=Tahyna, C=Calovo)

Flow sheet, 1)

Table 2

Collections of mosquitoes outdoors in the Seewinkel in 1967 and virus isolation experiments

	Date of Collection				
Species	May 5 - 7	May 19 - 21	June 2 - 4	June 17 - 18	June 30 - July 2
An.maculipennis	4/ 4/2/-*)	837/837/17/-	400/400/8/-	29/ 27/ 4/-	610/ 610/ 17/-
A.claviger	20/ - /-/-		1/ 1/1/-	55/ 15/ 3/-	13/ 12/ 2/-
A.plumbeus					
A.algeriensis				1/ -/ -/-	
Ae.flavescens	136/136/3/-	1/ 1/ 1/-	7/ 7/1/-	78/ 78/ 7/-	109/ 109/ 9/-
Ae.caspius/dorsalis	177/138/3/-			3052/2932/ 59/1T	5045/5042/ 102/13T
Ae.cantans		1/ 1/ 1/-			7/ 7/ 1/-
Ae.annulipes		10/ -/ -/-	12/ -/-/-		
Ae.leucomelas	1/ -/-/-				
Ae.sticticus				18/ 18/ 2/-	1/ 1/ 1/-
Ae.cinereus	1/ 1/1/-				1/ 1/ 1/-
Ae.vexans				467/ 464/ 14/-	293/ 283/ 10/-
M.richiardii			1/ 1/1/-	116/ 113/ 6/-	3803/3803/ 80/-
Th.annulata	8/ 8/2/-	5/ 5/ 1/-	124/124/3/-	25/ 25/ 3/-	23/ 23/ 3/-
U.unquiculata					
C.pipiens	11/ 11/2/-		5/ 5/1/-	27/ 13/ 1/-	15/ 15/ 2/-
C.modestus					
Unidentified				2600/2600/ 52/-	
	358/298/13/-	854/844/20/-	550/538/15/-	6468/6285/151/1T	9920/9906/ 228/13 T

Flow sheet, contd.2)

Table 2

Collections of mosquitoes outdoors in the Seewinkel in 1967 and virus isolation experiments

Species	Date of collection			
	July 14 - 17	July 24 - 26	August 4 - 6	August 18 - 20
An.maculipennis	754/ 748/ 16/-*)	2814/ 2814/ 62/1T	130/ 130/ 3/-	45/ 45/ 2/1C
A.claviger	6/ 6/ 1/-	86/ 86/ 4/-	2/ 2/ 1/-	
A.plumbeus				
A.algeriensis				
Ae.flavescens	10/ 10/ 1/-	2/ 2/ 2/-	2/ 2/ 1/-	
Ae.caspius dorsalis	172/ 172/ 4/-	2057/ 2057/ 43/-	197/ 195/ 5/-	177/175/ 5/-
Ae.cantans	1/ 1/ 1/-			
Ae.annulipes				
Ae.leucomelas				
Ae.sticticus				
Ae.cinereus				
Ae.vexans	16/ 16/ 2/-	111/ 111/ 7/-	139/ 138/ 4/-	4/ 4/ 2/-
M.richiardii	4001/ 4000/ 80/-	18507/18507/ 373/1T	772/ 772/16/-	331/330/ 8/-
Th.annulata	25/ 25/ 1/-	34/ 34/ 4/-		4/ 4/ 1/-
U.unquiculata				
C.pipiens	26/ 26/ 1/-	20/ 20/ 2/-	17/ 17/ 1/-	34/ 30/ 2/-
C.modestus	2/ -/ -/-		2/ 2/ 1/-	170/170/ 5/-
Unidentified	9100 9100/182/2T			
	14113/14104/289/2T	23631/23631/ 503/2T	1261/1258/32/-	765/758/25/1C

Flow sheet, contd.3)

Table 2

Collections of mosquitoes outdoors in the Seewinkel in 1957 and virus isolation experiments

Species	Date of Collection September 1 - 3	September 8 - 10	September 28 - 29	October 16 - 17
An.maculipennis	6/ 6/1/- *)	1/ 1/ 1/-	5/ 5/1/-	4/ 4/1/-
A.claviger				
A.plumbeus		1/ -/ -/-		
A.algeriensis				
Ae.flavescens	1/ 1/1/-			7/ 7/1/-
Ae.caspius dorsalis	12/ 12/1/-	5/ 5/ 1/-	1951/1943/30/-	131/ 130/3/-
Ae.cantans				
Ae.annulipes				
Ae.leucomelas				
Ae.sticticus				
Ae.cinereus				1/ 1/1/-
Ae.vexans				2/ 2/1/-
M.richiardii	277/277/6/-	273/273/ 6/-	59/ 59/7/-	
Th.annulata	3/ 3/1/-	5/ 5/ 1/-	49/ 46/1/-	
U.unquiculata	1/ -/-/-		10/ 9/1/-	
C.pipiens	40/ 40/1/-	4/ 4/ 1/-	275/ 272/6/-	250/ 250/5/-
C.modestus	46/ 46/1/-	4/ 4/ 1/-	12/ 9/1/-	1/ 1/1/-
Unidentified				
	386/385/12/-	293/292/11/-	2361/2343/56/-	395/395/13/-

Flow sheet, contd.4)

Table 2

Collections of mosquitoes outdoors in the Seewinkel in 1967 and virus isolation experiments

Species	October 29 - 30	November 12 - 13	Total
An.maculipennis	1/ -/ -/-*)	2/ 2/ 1/-	5642/ 5633/ 136/ 1 T, 1 C
A.claviger			183/ 122/ 12/ -
A.plumbeus			1/ -/ -/ -
A.algeriensis			1/ -/ -/ -
Ae.flavescens			353/ 353/ 27/ -
Ae.caspius dorsalis	10/ 10/ 1/-		12986/12811/ 272/ 14 T,
Ae.cantans			9/ 9/ 3/ -
Ae.annulipes			22/ -/ -/ -
Ae.leucomelas			1/ -/ -/ -
Ae.sticticus			19/ 19/ 3/ -
Ae.cinereus			3/ 3/ 3/ -
Ae.vexans			1032/ 1018/ 40/ -
M.richiardii			28140/28135/ 583/ 1 T,
Th.annulata	6/ 6/ 1/-	45/ 45/ 1/-	356/ 353/ 23/ -
U.unguiculata	1/ -/ -/-		12/ 9/ 1/ -
C.pipiens	427/424/ 9/-	197/197/ 4/-	1348/ 1324/ 38/ -
C.modestus			237/ 232/ 10/ -
Unidentified			11700/11700/ 234/ 2 T,
	445/440/11/-	244/244/ 6/-	62045/61721/ 1385/ 18 T, 1 C

*) Number of mosquitoes collected/Number of mosquitoes tested for virus/Number of pools/ Number of virus strains isolated (T=Tahyna, C=Calovo)

(3) Survey with sera of vertebrates

Table 1 gives an account of a serological survey done with sera of several vertebrate species occurring in the Seewinkel.

The sera were tested in the tissue culture neutralization test employing the established cell line GMK-AH 1. The cells were grown in tubes using Eagle's minimal essential medium (MEM) supplemented with 20 % calf serum as growth medium. Prior to inoculation, the medium was removed and replaced by Eagle's medium containing 5 % calf serum.

The serum samples were diluted 1:5 in PBS and tested against 30-300 TCM_{50} of Tahyna and Calovo viruses. The virus-serum mixture was incubated over night at + 4°C. For each serum two tubes were used. Maintenance medium was changed 24^h after inoculation and then, if necessary, at two days' intervals.

It must be stated that hedgehogs *(Erinaceus europaeus)* were investigated by mark and release-trapping using water-proof paints for marking. This study was done to investigate the possibility of overwintering of Tahyna virus in these heterothermal animals (see also page 66) and lasted from the beginning of 1967 until the end of 1968. During this period, a total of 147 individuals was marked; 116 hedgehogs were caught twice, 5 three times, 1 four times, 2 five times, and 1 six times. Conversions of antibodies against Tahyna virus were found in two individuals only, one in the period from May 5, 1967 and July 25, 1967, the other between July 15, 1967 and April 18, 1968.

It will be noted in Table 3 that most of the sera tested against Calovo virus were devoid of antibodies. Positive sera were only found among samples deriving from roe deer *(Capreolus capreolus)* and horses.

With Tahyna virus, the highest proportion of positive sera was present among hares *(Lepus europaeus)* and pigs.

Table 3

Serological survey on vertebrates occurring in the Seewinkel

Species	Number of sera collected	So far tested against Calovo virus/positive	So far tested against Tahyna virus/positive
Erinaceus europaeus	147	147/0	146/9
Sorex araneus	13	7/0	13/0
Crocidura leucodon	15	8/0	15/0
Crocidura suaveolens	2	-	2/0
Plecotus austriacus	1	-	1/0
Pipistrellus nathusii	1	-	1/0
Lepus europaeus	269	268/0	269/76
Citellus citellus	98	29/0	98/1
Cricetus cricetus	40	40/0	40/0
Pitymys subterraneus	2	2/0	2/0
Microtus arvalis	79	57/0	79/0
Apodemus flavicollis	15	11/0	15/0
Apodemus microps	22	21/0	22/0
Mus musculus	1	1/0	1/0
Vulpes vulpes	7	7/0	7/1
Sus scrofa	3	2/1	3/1
Sus scrofa domestica	17	17/0	17/11
Capreolus capreolus	10	10/7	9/4
Equus caballus	2	2/2	2/1
Lacerta agilis	65	-	65/0
Natrix natrix	7	7/0	7/0

(4) Studies on the periodicity of occurrence of Tahyna and Calovo viruses by means of sentinel rabbits

From spring until autumn 1967 and 1968, sentinel rabbits were exposed in two cages easily accessible for mosquitoes. The cages were placed in a distance of about 100 m from each other in the steppe biotope south of the village Apetlon in the Seewinkel-area. Blood was taken by heart puncture usually every two weeks. A small part of the blood was immediately frozen in dry ice and then kept at -80°C until inoculation into baby mice for virus isolation experiments. In case of virus isolation, strains were identified by means of the immunofluorescent method. The rest of the blood was used for serological studies. The sera were tested against Tahyna virus and Calovo virus in the NT as described elsewhere (see page 59).

So far, all blood samples obtained in 1967 were tested for virus. One strain of an agent was isolated on May 20 from rabbit 591 which could not be identified as Calovo nor as Tahyna virus. Studies on this agent will be carried out later. Only a small proportion of the blood samples obtained in 1968 were tested for virus. To date, two strains of Tahyna virus were isolated from rabbits bled on June 18.

In 1967, conversions of antibodies were observed against Tahyna virus with seven rabbits between July 1 (first specimens positive) and July 15 (last specimens positive). Conversions of antibodies against Calovo virus were found with four rabbits between July 15 and October 16.

In 1968, 11 rabbits developed antibodies against Tahyna virus with the first conversions in the serum samples taken on June 18 and the last on July 10. Tests with Calovo virus have not yet been done.

(5) Experimental studies on hibernation of Tahyna and Calovo viruses

5.1: Tahyna virus in experimentally infected frogs, lizzards and snakes.

In order to investigate the question of whether Tahyna virus can overwinter in poikilothermal vertebrates studies on the course of experimental infection in those species which are abundant in the Seewinkel area were carried out: two frog-species (Rana esculenta and Hyla arborea), one lizzard (Lacerta agilis)and one snake (Natrix natrix). Besides these species a few other amphibians and reptiles occur in the Seewinkel which are, however, rare and can be excluded for quantitative reasons.

5.1.1: Rana esculenta.

Two groups each comprising 12 adult frogs were injected subcutaneously with 50,000 LD_{50} or 50 LD_{50}, respectively, of a baby mouse brain suspension of Tahyna virus. The frogs were kept at + 22 ^{0}C. On the 3rd, 8th, 10th, 13th, 17th, 21st, 24th, 28th, 35th, 41st and 52nd day after infection one frog of each group was killed and tested for virus. Brain, heart, lungs, gall bladder, pancreatic gland, liver, kidneys, reproductive organs, fat body and spleen were ground, suspended in 1.5 ml of the medium described elsewhere in this report (see page 49) and centrifuged. The supernatant was inoculated intracerebrally into baby mice. No virus could be isolated, so that it appears that Rana esculenta does not develop viremia after infection with Tahyna virus.

5.1.2: Hyla arborea.

210 adult individuals of Hyla arborea were collected from trees and divided into four groups. Two groups each comprising 90 individuals were inoculated with 10,000 LD_{50} (group A) and 10 LD_{50} (group B), respectively, of a baby mouse brain suspension of Tahyna virus; two groups, each comprising 15 individuals, were injected with 1,000 LD_{50} (group C) and 100 LD_{50} (group D), respectively. One day after infection, 10 individuals of group A and group B were transferred to + 4 ^{0}C, the rest remained at a temperature of + 22 ^{0}C. From this stock, three individuals of group A and group B were killed on the 2nd, 6th, 8th, 10th, 15th, 17th, 20th, 22nd, 30th, 35th, 48th and 56th day after infection and tested for virus. Blood, heart, lungs and kidneys

were processed into one pool and suspended in 0.5 ml of the usual medium. After centrifugation, the suspension and a ten-fold dilution were inoculated intracerebrally into baby mice. No virus was isolated.

72 days after infection all remaining individuals of all four groups kept at + 22°C were bled. The sera were tested in the NT against Tahyna virus (method see page 59). No antibodies could be detected.

Those frogs kept at + 4°C were killed 133 days after infection and bled. No virus was isolated by intracerebral inoculation of the blood into baby mice nor could antibodies be detected against Tahyna virus.

From this it appears that Hyla arborea develops neither viremia nor antibodies after infection with Tahyna virus.

5,1,3: *Lacerta agilis.*

Two experiments were carried out using two different strains of Tahyna virus, a neuroadapted strain and an extraneural strain.

Experiment 1:

Two groups of adult lizzards, each comprising 12 individuals, were injected subcutaneously with 50,000 LD_{50} and 50 LD_{50}, respectively, of a baby mouse brain suspension of Tahyna virus. On the 2nd, 3rd, 4th, 6th, 8th, 10th, 13th, 15th, 17th, 20th and 23rd day after infection, one individual of each group was killed and bled. The blood was inoculated (undiluted and in a dilution of 10^{-1}) intracerebrally into baby mice. No virus was isolated.

In addition, five individuals were infected with 1.000,000 LD_{50}, five individuals with 100,000 LD_{50} and four individuals with 1,000 LD_{50} of the neuroadapted strain. All these lizzards were bled 78 days after infection. The sera were tested in the NT against Tahyna virus. No antibodies could be detected.

Experiment 2:

Two groups of lizzards each consisting of eight individuals were infected with 25,000 LD_{50} and 2,500 LD_{50}, respectively, of an extraneural strain of Tahyna virus obtained from viremic hamsters. The lizzards were kept at 22°C. On the 3rd, 4th, 7th, 11th, 21st and 42nd day after infection, one individual of each group was killed, and the

blood was inoculated intracerebrally into baby mice (undiluted and 10^{-1}). No virus was isolated. In addition, three individuals infected with 25,000 LD_{50} were transferred to + 4 °C four days after infection. They were blod 42 days after infection. Also in this case no virus was isolated. Thus, also lizzards failed to develop viremia and antibodies after infection with Tahyna virus.

5.1.4: Natrix natrix

Two groups of snakes, 10 adult individuals each, were injected with 25,000 LD_{50} and 2,500 LD_{50}, respectively, of an extraneural strain of Tahyna virus obtained from viremic hamsters. The snakes were kept for four days at + 22 °C. Then three individuals infected with 25,000 LD_{50} (group A) and three individuals infected with 2,500 LD_{50} (group B) remained at room temperature, the rest (group C and D) was transferred to + 4 °C.

On the 3rd, 4th, 7th, 11th, 14th, 21st, 38th, 81st and 119th day after infection blood was taken by clipping the tail from one snake of group A and group B and inoculated undiluted and in a dilution of 10^{-1} into baby mice. No virus was isolated.

The blood of one snake of group C and group D was tested ib the 38th and 119th day after infection. Also in these cases no virus was isolated.

All snakes of groups C and D were removed on the 119th day after infection and transferred to room temperature. Blood was taken on the 2nd, 3rd and 7th day after this removal (i.e.on the 121st, 122nd and 126 th day after infection) from one snake of both groups and inoculated into baby mice. No virus was isolated. On the 155th day after infection the blood of three snakes of each group was tested in the NT. No antibodies against Tahyna virus were detected.

From these results it can be concluded that the snake-species Natrix natrix does not show any signs of virus replication such as viremia or the production of neutralizing antibodies after inoculation with Tahyna virus.

5.2: Calovo virus in artificially infected Anopheles maculipennis.

As it has been demonstrated by many virus isolations Anopheles maculipennis is the main vector of Calovo virus

maintaining the virus cycle during the summer months. However, it is unknown whether the virus can also hibernate in this mosquito species which overwinters in the imaginal stage. This question was investigated in an experimental study.

In the period from October 6 to December 12, 1968, we collected about 6,000 overwintering females of *Anopheles maculipennis messeae* in hay barns. From these, 3,140 individuals were infected intrathoracally with Calovo virus (10^{-1} suspension of baby mouse brain in PBS containing 10 % calf serum).

After inoculation, most of the mosquitoes were incubated at + 22 °C and 90 % r.h. for five days and then kept at + 4 °C and 90 % r.h. Only a small part was kept in the attic under conditions similar to those in nature with varying temperature and humidity. Six hours, 24 hours, 4,5,25,32,40,42,46,53,60,67,119,126,133,147 and 155 days after infection three mosquitoes were removed, suspended in 1.5 ml of TCM 199 containing 0.75 % bovine albumine and antibiotics, buffered to 7.2 pH, and titrated in baby mice by intracerebral infection. After 5 months, the supply of surviving mosquitoes was completely exhausted.

The results of these virus isolation experiments are shown in Table 4.

From these results it appears that replication of the virus starts between the 1st and 4th day after inoculation. After two months the virus titer decreases slowly. Yet even after 5 months, when the last surviving mosquitoes were tested, virus was still detectable.

Out of the small numbers of infected mosquitoes kept under more or less natural conditions in the attic three individuals were tested for virus on the 32nd and 115th day after inoculation. Both pools were positive.

(6) Discussion

Tahyna virus

During the last three years, Tahyna virus regularly occurred in the steppe biotops in the Eastern part of the Neusiedlersee-area. There, the main vectors are Aedes caspius and Aedes dorsalis in which very high infection rates were observed; besides by these species the virus is also transmitted by Aedes flavescens and Mansonia richiardii. Three strains were isolated from Anopheles maculipennis, but these strains probably derived from individuals newly engorged on viremic cattle. As could be demonstrated by Danielova (6), the virus does not multiply in Anopheles maculipennis. In the lowlands along the rivers the virus is mainly transmitted by Aedes vexans and Aedes cantans (2,7,8).

From all these mosquito species virus was isolated in June, July and August only. These findings are in good agreement with the results obtained with sentinel rabbits.

From the results of our serological studies it can be concluded that hares and roe deer are the main vertebrate hosts, while pigs might maintain the cycle in the villages. In addition, antibodies were found in hedgehogs, ground squirrels, foxes, wild boars and horses.

Thus, the main arthropod and vertebrate hosts which maintain the virus cycle during summer are known.

The mode of hibernation is, however, still unknown. From our serological studies with hedgehogs carried out on the basis of mark and release-trapping it appears that this hetero-thermal vertebrate species does not maintain the virus cycle during winter, though a prolonged viremia could be demonstrated in the cold under experimental conditions (9). Serological surveys carried out with hamsters, ground squirrels, lizzards and snakes have also yielded negative results. Frogs (Rana esculenta, Hyla arborea), lizzards (Lacerta agilis) and snakes (Natrix natrix) do not develop viremia nor antibodies after inoculation of virus, so that it can be concluded that they do not act as hosts of the virus. Thus, a hibernation in those species can also be excluded.

Danielova et al. (10) have recently studied the possibility of hibernation of Tahyna virus in overwintering females of Culiseta annulata. Under experimental conditions,

the virus could be isolated up to 12 weeks after infection. *Culiseta annulata* is, however, a rather rare species, so that it seems unlikely that the low population densities are sufficient for the maintenance of the virus cycle, if even the virus can overwinter in this mosquito under natural conditions.

It is striking that the seasonal appearance of Tahyna virus coincides with the development of Aedes species, mainly of *Aedes caspius* and *Aedes dorsalis*. One wonders, therefore, whether transovarial infection of the mosquito vectors is possible. So far, transovarial infection in mosquitoes has not yet been established with any virus of the arbo group (Chamberlain 11). Future investigations should, however, deal with this problem in detail. It might be possible that the virus is transmitted in an uninfectious form to the eggs and to the following instars, becoming infective in the imaginal stage through the influence of mutagenic noxes. As the rearing of the vectors of the Tahyna virus meets many technical difficulties, these studies should first be conducted with a model, for example *Aedes aegypti*, using Tahyna virus and a virus which is transmitted by *Aedes aegypti* in nature such as Dengue.

Calovo virus

It became evident through our studies that in the steppe biotops east of the Neusiedlersee at the Hungarian border the Calovo virus occurs - at least in some years - in unusually high infection rates. The main vector of this virus is undoubtedly *Anopheles maculipennis*; besides this species, only *M. richiardii* was occasionally found to be infected. All virus isolations were made from mosquitoes collected during August and September only. This roughly corresponds with the results obtained with the sentinel rabbit technique. In a two years's study conversions of antibodies against Calovo virus were invariably observed during the period from the middle of July until the middle of October.

In a serological survey, roe deer, horses and wild boars were found to have antibodies against the virus. It is not yet quite clear which vertebrate species maintains the cycle. Besides, roe deer which are fairly abundant in the area under investigation also cattle which is heavily attacked by *Anopheles maculipennis* has to be considered as host of this virus. This is indicated by the fact that most of the strains were isolated from mosquitoes collected in a cow barn. A serological survey will clarify this point.

All sera of heterothermal and poikilothermal vertebrates (hedgehogs, hamsters, ground squirrels, lizzards and snakes) tested for antibodies against Calovo virus were found to be negative. A study on the course of experimental infection of snakes with Calovo virus is still in work. From the results so far obtained it may, however, be concluded that heterothermal and poikilothermal vertebrates do not take part in the virus circulation and, particularly, do not act as hosts during winter.

In order to investigate whether the virus can hibernate in overwintering females of Anopheles maculipennis messeae, the course of experimental infection with Calovo virus by intrathoracal infection of this species was studied. From the results it appears that virus replication starts between the first and fourth day after infection. In the course of five months a slow decrease of virus titer in the mosquitoes was observed which may be traced back to the reduced physiological status during hibernation. It is, however, conceivable that the virus titer would increase after a blood meal. Danielova et al. (10) who infected overwintering females of Culiseta annulata with Tahyna virus also observed a decrease of the virus titer during hibernation,but found higher titers in mosquitoes which had been exposed to higher temperature after interrupting the hibernation before the virus isolation experiment. The fact that the Calovo virus could be demonstrated in overwintering females of Anopheles maculipennis even five months after infection leads to the assumption that the virus might possibly hibernate in Anopheles maculipennis. It is, however, striking that signs of virus activity in nature could never be detected before the middle of August. On the other hand this discrepancy might be explained by the low numbers of infected mosquitoes surviving until spring. The infection rates might be very low in spring and slowly increase until reaching a detectable level in summer.

In future studies it should be explored if virus replication in hibernating females of Anopheles maculipennis can be stimulated by means of a blood meal. In addition, the possibility of a transovarial infection should be studied in detail. For this purpose, females of Anopheles maculipennis collected in the field should be infected, and the F_1-generation should be exposed to mutagenic noxes thus possibly inducing a tranformation of the "ecliptic phase" of the virus into an infectious form.

(7) Summary

Field investigations and experimental studies on the ecology of Tahyna and Calovo viruses were carried out. Both viruses occur in high infection rates among certain species of mosquitoes in the eastern part of the Neusiedlersee-area near the Hungarian border. The main vectors of Tahyna virus are _Aedes caspius_ and _Aedes dorsalis_, the Calovo virus is mainly transmitted by _Anopheles maculipennis_. Virus isolations from mosquitoes and virological and serological studies done with sentinel rabbits have shown that Tahyna virus is detectable from the middle of June until the end of August, and Calovo virus from the middle of July until the middle of October.

The main vertebrate hosts of Tahyna virus are hares, roe deer and pigs. Antibodies against Calovo virus were found in roe deer and horses only.

The hibernation of both viruses is still unknown. It was established that neither heterothermal (hedgehogs, ground squirrels, hamsters) nor poikilothermal vertebrates (frogs, lizzards, snakes) can maintain the virus cycles during winter. Due to the fact that the Calovo virus replicates in hibernating females of _Anopheles maculipennis_ after intrathoracal infection and was detectable even after 5 months, evidence suggests that this virus might perhaps overwinter in this mosquito.

Table 4

Virus isolation experiments from overwintering <u>Anopheles maculipennis messeae</u> infected with Calovo virus

Hours resp. days after infection	Number of mice infected/Number of mice died 3 mosquitoes in 1,5 ml of medium	10^{-1}	10^{-2}	10^{-3}	10^{-4}
6 hours	8/8	5/2	7/3	6/0	5/0
24 hours	7/7	8/6	5/0	8/0	5/0
4 days	7/7	7/7	8/8	6/4	8/1
5 days	8/8	5/5	5/5	6/1	6/1
25 days	9/9	9/9	6/4	6/1	10/0
32 days	6/6	5/5	7/5	5/0	7/0
40 days	5/5	5/5	6/3	8/2	8/0
42 days	7/7	5/5	6/6	5/3	8/5
46 days	7/7	8/7	8/5	6/1	-
53 days	9/9	6/6	10/8	8/0	7/0
60 days	8/8	10/0	7/0	7/0	6/0
67 days	9/9	9/8	8/1	8/0	7/0
119 days	8/7	8/0	8/0	8/0	8/0
126 days	9/7	9/3	7/0	7/0	6/0
133 days	8/8	10/6	6/1	8/0	
147 days	10/2	9/1	8/0	8/0	
155 days	9/5	8/2	5/0	7/0	

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INVESTIGATIONS ON THE AGENT OF HEMORRHAGIC FEVER IN GERMANY ("MARBURG VIRUS", VERVET MONKEY DISEASE AGENT, RHABDOVIRUS SIMIAE) AND ITS POSSIBLE CLASSIFICATION AS AN ARBOVIRUS

(1) Introduction

In last year's report (1) we summarized the results of a study on the so-called "Marburg virus". This hitherto unknown agent caused an outbreak of hemorrhagic fever among laboratory workers in Marburg and Frankfurt (Germany) in August and September 1967. We reported that the agent shows some characteristics of an arbovirus. It replicates in the brains of baby mice, is sensitive to desoxycholate and its morphology resembles those of the rod-shaped arboviruses which are now placed in the rhabdovirus group. Other workers found that the agent is inactivated by ether (2) and contains RNA (3) thus exhibiting features which are also compatible with the arbovirus group.

In 1968, our research unit carried out intensive studies with this fascinating new virus. In particular, we attempted to get some more information on the question whether or not it is justified to classify the agent as an arbovirus. The term "arbovirus" is presently used as an ecologic criterium. In this sense also the mosquito-borne rhabdoviruses such as Vesicular Stomatitis, Hart Park and Cocal viruses are members of the arbovirus group.

(2) Attempts at the propagation in arthropods

In a first series of experiments we investigated the ability of the virus to multiply in artificially infected arthropods.

(2.1) Ticks of Species Ixodes ricinus

Female ticks were infected by the anal route with a thin glass capillary filled with viremic guinea pig serum containing $10^7 LD_{50}$/ml. Infected ticks were kept in a moist atmosphere at room temperature for 18 days at which time the survivors were ground and suspended in PBS. This suspension was injected into ticks for a second passage and into guinea pigs to test for virus. Ticks of the second passage were also tested for virus after 15 days in guinea pigs. No virus could be isolated from guinea pigs infected with a suspension of the initially infected

ticks or those of the second passage. Therefore, we concluded that the "Marburg virus" does not replicate in *Ixodes ricinus.*

(2.2) Mosquitoes of the Species *Aedes aegypti*

For investigating the ability of the "Marburg virus" to replicate in mosquitoes, we first employed *Aedes aegypti* mosquitoes which is the most commonly used species for this purpose.

The mosquitoes were injected intrathoracally with the serum of a viremic guinea pig by a very thin glass capillary. The infected *Aedes* were kept in an incubator at 26°C and fed with honey water. A high humidity was maintained at all times. On the 11th day surviving mosquitoes were ground and suspended in PBS. The suspension was injected into guinea pigs and also into new *Aedes* for a second virus passage. Mosquitoes of the second passage were sacrificed 21 days after infection and tested for virus in guinea pigs.

From both passages, virus could be reisolated in guinea pigs. Specifity of the reisolated agent was confirmed by means of fluorescein-labelled antibodies.

Thus, we concluded that "Marburg virus" replicates in *Aedes aegypti*, which is a member of the subgroup *Culicinae* from the *Culicidae* group of arthropods.

(2.3) Mosquitoes of the Species *Anopheles maculipennis*

For investigating whether "Marburg virus" also propagates in mosquitoes from the subgroup Anophelinae, *Anopheles maculipennis* mosquitoes were also injected intrathoracally with the virus-containing serum. Infected mosquitoes were kept at 22°C and high humidity. On the 2nd, 4th, 8th and 14th day, respectively, 10-20 individuals were ground, suspended in PBS and tested for virus by injecting the suspension into guinea pigs. Virus was reisolated after 2 and 4 days; only a low concentration of virus was demonstrable after 8 days but none after 14 days. From this it is apparent that "Marburg virus" persisted for a few days in *Anopheles maculipennis* but did not replicate in this mosquito species.

Due to its successful propagation in *Aedes aegypti* the "Marburg virus" corresponds to the ecologic definition of an "Arbovirus". The fact that the virus failed to

replicate in Anopheles maculipennis suggests that it is possibly transmitted in nature by mosquitoes (Culicidae) of the subfamily Culicinae.

Summary

It was attempted to propagate the "Marburg virus" in three different species of arthropods: Ixodes ricinus ticks and mosquitoes of the species Aedes aegypti and Anopheles maculipennis. No virus replication was observed in Ixodes ricinus and in Anopheles maculipennis. However, the virus multiplied in Aedes aegypti. Therefore, the "Marburg virus" corresponds to the ecologic definition "Arbovirus" and is possibly transmitted in nature by mosquitoes of the subfamily Culicinae of which Aedes aegypti is a member.

(3) Formation of interferon in the brain of baby mice after infection with "Marburg virus" and some Rhabdoviruses

The mosquito-borne rhabdoviruses: VSV, Cocal and Hart Park were tested for their ability to produce interferon in baby mouse brain. Due to the similarity of the "Marburg virus" to these viruses (see page 26) this agent was incorporated in the study.

Baby mice were infected with high doses of these viruses. When the animals infected with Cocal, Hart Park and VSV, were in a moribund state, the brains were removed. After Marburg virus-infection the white baby mouse shows no signs of illness (1). Therefore brains of mice infected with this virus were harvested after different days and at different levels of passage (see Table 1). The brains were suspended in distilled water and dialysed against citrate buffer pH 2, phosphate buffer pH 7.5 and distilled water. Eagle's medium of 5-fold concentration was added 1:4. Two-fold dilutions of these preparations were tested to prevent infection of 100-300 $TCID_{50}$ of EMC virus in L cells with the method described by Vilcek and Stancek (4). As can be seen in Table 1 no production of interferon was observed after infection with "Marburg virus". However, suspensions of brains infected with VSV, Cocal and Hart Park inhibited the challenge virus infection in a dilution of 1:40, 1:80 and 1:640, respectively. Thus giving rise to the production of high levels of interferon in baby mouse brain is no common feature of the rhabdovirus group.

Summary

Low titers of interferon were found in brains of baby mice infected with VSV (1:40) and Cocal virus (1:80). A high titer of interferon (1:640) was found in baby mouse brain after infection with Hart Park virus. However, in brains of baby mice infected with the "Marburg virus" no interferon was detectable.

(4) Influence of Poly I:C on the experimental infection in hamsters

In this study the influence of the interferon-stimulating drug Poly I:C (Miles Chem.Corp.) on the experimental infection with the "Marburg virus" was investigated. This drug which is a compound of polyriboinosinic acid and Polyribocytidylic acid is capable of protecting mice against fatal infection with TBE virus (see page 28).

Twenty-seven young hamsters weighing 12-15 g were treated with 100 µg Poly I:C, 18 and 3 hours before intracerebral (0,02 ml) infection. For infection, a dilution of 10^{-3} of infected baby hamster liver was used. Prior to infection the "Marburg virus" had undergone three passages in baby hamsters. For control purposes 27 animals were infected but not treated. Four untreated hamsters were inoculated with a virus dilution of 10^{-4} for titrating the virus dose.

From the hamsters treated with Poly I:C 14 died and from the control group 13 succumbed the infection . The average survival time, 7.79 and 8.85 days respectively, did not show any marked differences. From this, it was obvious that Poly I:C gave no protection against the "Marburg virus" infection.

Summary

The interferon-inducing drug Poly I:C had no influence on the experimental infection of hamsters with the "Marburg virus".

(5) Propagation in various tissue cultures

In a previous study, we assessed the susceptibility of various primary cell cultures and permanent cell lines for the propagation of "Marburg virus" (5). Although the virus multiplied in a variety of cell tissue cultures, no CPE was observed. Therefore, we contineud our studies

with the aim to find tissue culture cells which would give a clear-cut CPE.

Stock cultures of L (mouse embryo) and U (human amnion) cells were grown in French square bottles in a medium consisting of 90 % Eagle's minimum essential medium (MEM) made up with Hank's balanced salt solution and 10 % calf serum. For viral inoculation cells were grown in tubes and, after the cell sheet was complete, the medium was replaced by a maintenance medium containing 95 % Eagle's medium and 5 % calf serum.

After inoculation fluids were changed on the first and on the third day. On the fifth day the supernatant was inoculated into guinea pigs for assessment of virus replication.

The results, given in Table 2, show that the virus did not multiply in L cells. By contrast, a virus replication in U cells could be demonstrated in which three virus passages were done. However, the virus did not produce a CPE.

Recently we came across the stable ELF (Human embryonic lung fibroblasts) cell line which we also tested. The cells were grown in Eagle's medium as described above using 10 % fetal bovine serum instead of calf serum; then, for inoculation purposes, the cultures were maintained with Eagle's medium supplemented with 10 % calf serum. After infection the tissue culture fluid was changed daily.

As indicated in Table 2 in these cells "Marburg virus" produces a marked CPE, which appears about the third day and reaches its maximum about the fifth day after infection. CPE begins in focal areas and consists of spindling and later on of clumping of cells. Finally the foci become confluent (see Fig.1 and 2). It must be mentioned, that although destructions are extensive, they are never complete and eventually healthy cells may grow in and repair the lesions in the cell sheet. It can further be seen in Table 2 that the supernatant of a 4-day infected culture had a titer of about 10^4 $TCID_{50}$ when tested in the cells and of $10^{6.5}$ LD_{50} when assayed in guinea pigs. Thus this cell culture was less sensitive to the virus than guinea pigs. However, the virus gives a clear-cut CPE and endpoints are readily determinable, so that this cell line can be useful for further studies on the virus.

Summary

L cells did not allow any growth of "Marburg virus". U cells propagated the virus, but no CPE was seen. However, in ELF cells the virus replicated and a clear-cut CPE was observed which will be useful for further studies on the virus.

(6) Complement-fixing antibodies in the sera of patients with "Marburg virus" disease

During the outbreak of hemorrhagic fever we received serum samples from patients who were hospitalized with the disease either in Marburg or in Frankfurt. This gave us the opportunity to test the sera in the complement-fixation (CF) test.

A crude complement-fixing antigen of "Marburg virus" was prepared in the following manner: Infected livers of guinea pigs were homogenized and suspended 1:10 in veronal buffer pH 7.3. After centrifugation, the supernatant fluid was used as the antigen. Attempts to make handling of the antigen less dangerous by adding 0.3 % propriolaktone failed because the preparation became inactive.

CF tests were done with a drop-type technique previously described (6). Dilutions of the sera from 1:4 to 1:128 were tested against dilutions of antigen from 1:8 to 1:64. The results, which are summarized in Table 3, show that antibodies were detectable in sera of nine patients who had overt disease. Complement-fixing activities in sera appeared in the second week of illness and reached a maximal titer of 1:32 - 1:64 in the third or fourth week. This maximal titer seems to persist for only a few weeks, because in two sera drawn in the reconvalescence (60th-70th day of illness) titers already dropped to 1/2 or 1/8 respectively of the initial level.

It will be noted that the sera of three patients were negative in the CF test. These persons were at first thought to have "Marburg virus" disease but later were found to have had different illnesses. The results provide conclusive evidence that the CF test can be used for diagnosis of "Marburg virus" disease.

Summary

Sera of patients with "Marburg virus" disease were investigated in complement-fixation test using a crude

CF-antigen. Antibodies were detectable in sera of nine patients, while three other patients, who later were found to have had illnesses other than Marburg hemorrhagic fever, had no antibodies. Complement-fixing activities in the sera of patients appeared in the second week of illness and reached a maximum in the third or fourth week.

Table 1

Content of interferon in baby mouse brain after infection with "Marburg virus" and some rhabdoviruses

Virus	Mouse passage	Days p.i.	Titer of Interferon
VSV	2 (Vienna)	2	1 : 40
Cocal	4 (Vienna)	2	1 : 80
Hart Park	3 (Vienna)	5	1 :640
Marburg virus	1	5	<1 : 40
	1	8	<1 : 40
	1	16	<1 : 40
	5	5	<1 : 40
	18	5	<1 : 40

Table 2

Propagation of "Marburg virus" in three permanent cell lines

Cells	Growth medium	Maintenance medium	Number of passages	Appearance of CPE	Titer yielded in tissue culture fluid
L (Mouse embryo cells)	MEM 10% CS	MEM 5% CS	1	0	0
U (Human amnion)	MEM 10% CS	MEM 5% CS	3	0	n.d.
ELF (Human embryonic fibroblasts)	MEM 10% FBS	MEM 5% CS	4	3rd-5th day p.i.	10^4 t.c. $10^{6.5}$ g.p.

MEM = Minimum essential medium (Eagle)

CS = Calf serum

FBS = Fetal bovine serum

g.p. = Assayed in guinea pigs

t.c. = Assayed in tissue cultures

n.d. = Not done

Table 3

Results of complement-fixation tests with sera from patients with "Marburg virus" disease

Patient	Serum	Day of illness	Titer serum / Titer antigen
KL	1	4	1 : 8 / 16
	2	9	1 :16 / 64
	3	14	1 :64 > / 64
	4	20	1 :32 > / 64
	5	21	1 :32 > / 64
	6	28	1 :64 > / 64
HI	1	9	Ø
	2	12	1 : 4/16
	3	17	1 :16/16
	4	28	1 :32/64
FLA	1	20	1 :64/32
	2	23	1 :64/32
	3	31	1 :64:32
HA	1	14	1 : 4/32
	2	23	1 :64/ > 64
	3	60 - 70	1 :32/ > 64
MA	1	9	1 : 4/8
	2	17	1 :32/64
	3	60 - 70	1 : 4/16
GI	1	6	1 : 4/8
	2	14	1 :32/64
KR	1	23	1 :64/ > 64
MU	1	21	1 :32/32
UL	1	20	1 :64/ > 64
FL*	1	18	Ø
GÖ*	1	19	Ø
LI*	1	21	Ø

* = Febrile illness other than "Marburg virus" disease
Ø = < 1 : 4

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Figure 1

ELF cells inoculated with "Marburg virus" 5 days after infection

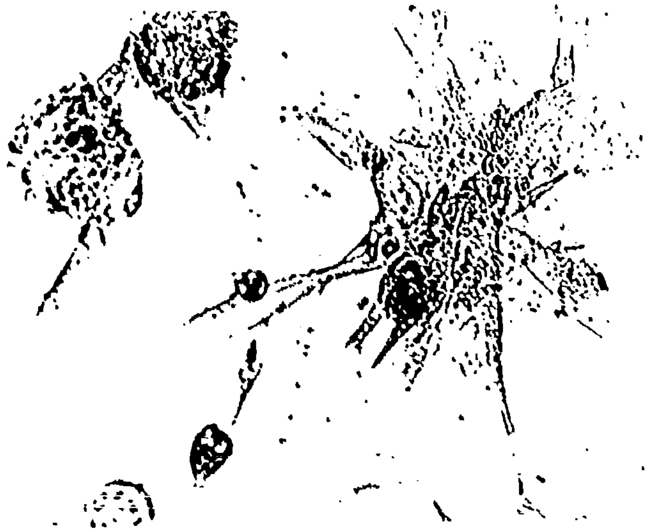

Figure 2

Uninoculated ELF cells

SEROLOGICAL INVESTIGATIONS ON DOMESTIC ANIMALS AND MICE FROM TURKEY

In 1965, an expedition to Anatolia, Turkey, sponsored by the Muserum of Natural History in Vienna, was carried out, in order to collect animals of different groups. Dr.A.Radda took part in this travel and he was able to collect blood samples from 214 domestic and free-living animals; these sera were tested for hemagglutination-inhibiting antibodies against several arboviruses.

Material and Methods

The sera were treated with acetone and tested in a dilution of 1 : 10 against 8 units of antigen. Positive sera were further diluted in two-fold dilutions.

The following sucrose-acetone treated antigens were used: Sindbis, Semliki, Tick-borne Encephalitis (TBE), West-Nile (WN), Murray Valley Encephalitis (MVE) and Dengue II (D II). In addition, positive sera were partially tested on monolayer L cells in the neutralization test (NT) with methods described elsewhere in this report (see page 2).

Results

The results of the serological survey can be seen in Table 1. Forty-nine sera from cattle deriving from different Vilayets (Turkish districts or counties) of central and eastern Anatolia and nine sera from free-living mice, caught in the western Anatolia, showed no antibodies against these viruses. From 45 sera from sheep deriving from the surroundings of Ankara (Numbers A 57-95 in Table 2), 11 sera showed antibodies against MVE virus. Nine of those positive sera were also positive against WN and 3 of them were positive against D II viruses. Out of 110 sera (Numbers H 10 and 104 in Table 2) from the slaughter-houses of Antakya (Vil Hatay) and Iskenderun (Vil Hatay), which were obtained mainly from sheep, but also from several goats and cattle, 3 showed antibodies against MVE and D II, two against TBE and one against WN. Two others were positive against Sindbis virus. Table 2 lists all positive sera including titers of HI antibodies and the results of the NT. The results of the serological survey using the HI-test were further proved by use of the NT. All positive HI sera inhibited CPE of cells exposed to the same virus.

Discussion

It is of great interest that animals from different parts of Anatolia showed antibodies against arboviruses of the groups A and B.

From our results we assume that in the surroundings of Ankara WN virus, or an agent very closely related, is active. Positive tests with MVE and D II antigens are probably due to cross reactions with these group B viruses.

In the Vilayet Hatay, which is situated in the South-east of Anatolia near the boundary to Syria, the activity of two arboviruses - one of group A and one of group B - could be demonstrated. From the results obtained with serum number 41 it seems to be a reasonable assumption that the group B agent is TBE virus. The results of the tests of serum number H 20 indicate that in the Hatay-Vilayet another group of B virus - perhaps West Nile virus - may be active.

Summary

Sera (214) of domestic animals and free-living mice from Anatolia were tested for HI antibodies against several arboviruses (Sindbis, Semliki, TBE, West Nile, MVE, Dengue II).

From these, all 49 sera of cattle and all 9 sera of mice showed no antibodies against these viruses. From the remaining 155 sera (mainly from sheep) 14 sera showed antibodies against MVE, 10 against West Nile ,6 against Dengue II, 2 against TBE and Sindbis. Some sera were positive against two or even three different viruses.

Table 1

Serological survey of animal-sera from Turkey

Number of sera	Animal species	Descent district	Number of HI-positive sera
49	Cattle	Central and eastern Anatolia	0
45	Sheep	Surroundings of Ankara	11 MVE/9 WN/ /3 D II
9	Mice	Western Anatolia	0
60	Sheep, goats, cattle	Antakya (Hatay)	3 MVE/3 D II/ / 2 TBE/1 WN
50	Sheep, goats, cattle	Iskenderun (Hatay)	2 Sindbis

Table 2

Results of HI and NT with animal-sera from Turkey

	West Nile HI	West Nile NT	MVE HI	D II HI	TBE HI	TBE NT	Sindbis HI	Sindbis NT	Semliki HI
A 57	1:20	(+)	1:10	-	-		-		-
61	1:10	(+)	1:10	-	-		-		-
62	-	(+)	1:10	-	-		-		-
64	1:40	(+)	1:40	1:10	-		-		-
65	1:20	(+)	1:20	-	-		-		-
73	1:20	(+)	1:20	1:10	-		-		-
77	1:10	(+)	1:10	-	-		-		-
79	1:10	(+)	1:10	-	-		-		-
90	-	(+)	1:10	-	-		-		-
93	1:10	(+)	1:10	1:10	-		-		-
95	1:10	(+)	1:10		-		-	(-)	-
H 10	-		1:10	1:20	1:10	(+)	-		-
20	1:20		1:20	1:20	-	(-)	-		-
41	-		1:20	1:20	1:160	(+)	-		-
42	-	(-)	-	-	-		1:20	(+)	-
104	-		-	-	-		1:20	(+)	-

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13. ABSTRACT

Three new foci of TBE virus were located in Upper Austria. Shrews are not essential for virus cycle in Lower Austria. Survey with sera of game showed that TBE foci are scarcer in the West than in the East of Lower Austria. HI test was as specific but less sensitive than the NT. Receptor-analogue substances for TBE virus are probably Ca- and Mg-salts of polyphosphoinositides. Different strains of TBE virus induced the same level of interferon in babymouse brain. Other viruses of the TBE complex gave slightly higher interferon titers. The interferon inducing compound Poly I:C exhibited excellent protection against infection with TBE virus in mice. Experimental studies showed that foxes, polecats and weasels can act as host of TBE virus. The main arthropod and vertebrate hosts of Tahyna and Calovo viruses were established. Neither heterothermal nor poikilothermal vertebrates can maintain the virus cycle in winter. Overwintering of Calovo virus in Anopheles maculipennis is conceivable. "Marburg virus" replicated in Aedes aegypti but failed to multiply in Anopheles maculipennis and in Ixodes ricinus. The virus did not induce formation of interferon in brains of baby mice and was not inhibited by Poly I:C. The agent produces CPE in ELF cells. CF test was found to be useful for diagnosis of "Marburg virus" disease. Animals from Anatolia had antibodies against arboviruses of groups A and B.

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